REVERSE TRANSCRIPTASE

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I. INTRODUCTION

A. Discovery

Recognition of RNA tumor viruses as causative agents of malignant disease started with the observation (by Ellerman and Bang in 1908) that a filterable agent transmitted avian leukemia[1] and the subsequent isolation of chicken sarcoma virus by Rous in 1911.[2] These findings were followed by an intense and continuous search for new isolates, the establishment of inbred strains of mice, and the development of tissue culture techniques. In the early 1960s, the significance of RNA tumor viruses was generally accepted, especially in avian and murine systems, but with some skepticism, probably because its role in natural disease was not yet evident. Moreover, the mode of replication of the RNA tumor viruses was still not understood. In recent years, the study of these viruses markedly intensified, principally because of a discovery which filled a major gap in the understanding of their replication. This is the independent discovery of the viral RNA-dependent DNA polymerase (reverse transcriptase) by Temin and Mizutani[3] and by Baltimore[4] in 1970. This discovery also extended our understanding of the mode of genetic information transfer, and it paved the way for the finding of viral-related components, reverse transcriptase[5-11] and type-C viral related nucleic acids in human leukemic cells by Gallo, Spiegelman, and their co-workers (see later section[10,12-17]).

In the late 1950s and early 1960s progress in the molecular biology of bacteria and the bacteriophages clearly showed that genetic information was transmitted from DNA to RNA and that genetic information was conserved in DNA. Due to its predictability, studies on DNA viruses such as polyoma and SV40 quickly benefited from the knowledge gained from DNA phages. At this time, studies on RNA tumor viruses had just turned from an emphasis on animal experiments to tissue culture. Investigations into the question of how an RNA tumor virus replicates and completes its life cycle was timely. Most studies were performed with avian leukosis sarcoma viruses and with murine leukemia-sarcoma viruses.

Generally, cells infected by RNA tumor viruses are not killed and the viral genetic information is transmitted to daughter cells at mitosis. The structure that transmits virus information is called the provirus. The first indication that DNA was involved in the reproduction of these viruses came

from some studies with inhibitors. It was observed that virus production was drastically reduced when infected cells were exposed to DNA synthesis inhibitors such as actinomycin D, 5-bromodeoxyuridine, 5-iododeoxyuridine, or cytosine arabinoside early after infection.[18-21] In fact, Temin demonstrated that in chicken fibroblasts transformed by Rous sarcoma virus, the provirus was a new DNA homologous to the RNA isolated from RSV as judged by molecular hybridization.[22] These preliminary findings led Temin to propose a DNA provirus hypothesis[23] in 1964. The hypothesis stated that the provirus is DNA and that the replication of leukovirus involved successive transfers of information from viral RNA to proviral DNA to progeny viral RNA, whereas the replication of the provirus accompanying cell multiplication involved information transfer from DNA to DNA. This hypothesis could explain most of the findings of Temin,[18,19] Bader,[20] and others, and it gained additional support from further studies. For example, Baluda and Nayak observed sensitivity of viral replication to actinomycin D and found a new DNA sequence homologous to viral RNA in the viral transformed cells.[24] The newly synthesized DNA was shown to be different from that synthesized in S-phase of the cell cycle.[25,26] It was observed that stationary cells exposed to RSV were stably infected, but virus production started only after cell division was initiated. While the stationary cells are generally resistant to DNA synthesis inhibitors, the establishment of a stable viral infection in these stationary cells is sensitive to the same DNA synthesis inhibitors. Furthermore, Balducci and Morgan[27] and Boettiger and Temin[28] showed that a stable infection of RSV was obtained by infecting stationary chicken fibroblasts in the presence of 5′-bromodeoxyuridine. This infection is aborted by exposing the infected cells to light and this effect is dependent on the multiplicity of infection. These results suggested that a bromodeoxyuridine-containing DNA with information required for the successful infection was synthesized in the infected cells and this DNA was a copy of the information from the input virus rather than a copy of cellular DNA.

With this convincing biological data, some Investigators (in addition to Temin) predicted a possible existence of a DNA polymerase which could transfer viral genetic information from RNA to DNA.[29,30] However, the possibility that double-stranded RNA was an intermediate in viral replication was not completely ruled out. In fact, attempts were made to find an RNA→RNA replicase in cells infected by an RNA tumor virus.[31]

In the late 1960s, following the isolation of sigma factor from purified *Escherichia coli* RNA polymerase by Burgess and his colleagues[32] and the finding of the induction of viral specific replicase in Qβ phage-infected cells,[33] many scientists began searching for nucleotide polymerases both in cells and in virions. The purpose was to understand gene regulation through elucidation of the specific transcription of the viral genome. At that time, vaccinia virus, a DNA virus, was found to contain a DNA-dependent RNA polymerase,[34,35] Reovirus, a double-stranded RNA virus, an RNA-dependent RNA polymerase,[36,37] and vesicular stomatitis virus, a single-stranded RNA virus, an RNA-dependent RNA polymerase.[38] With these findings it was logical to consider the existence of a replication enzyme in RNA tumor viruses. In fact, some other biological experiments provided an additional hint. It was found that the exposure of stationary cells to protein synthesis inhibitors such as puromycin or cycloheximide did not affect the formation of the provirus after infection by RSV.[39,40] This finding suggested that the enzyme required for the formation of proviral DNA was already present, i.e., it did not require protein synthesis. Finally, the viral RNA-dependent DNA polymerase was found in the virions of Rous sarcoma virus by Temin and Mizutani[3] and additionally in Rauscher leukemia virus by Baltimore,[4] findings quickly confirmed and extended to a number of other viruses by several groups (e.g., References 41 and 42).

B. After effects

RNA-dependent DNA polymerases were soon found in many RNA-containing viruses. At present all of the known infectious type-C virus, type-B virus, Mason-Pfizer monkey type virus, sheep slow virus, and foamy virus have been shown to contain this DNA polymerase. These are listed in Table 1. Since the procedure for assay of RNA-dependent DNA polymerases is relatively simple and the enzyme activity is relatively specific, this enzyme has become a useful tool for many purposes, e.g., for quantitating known virus, for studying the process of viral replication, for searching for the

TABLE 1

Particles Containing Reverse Transcriptase*

I. Extracellular Particles
  A. Type-C particles
    1. Leukemia-sarcoma viruses
      1) Mammalian leukemia-sarcoma virus
        Primate: SiSV, SiLV;[43,44] GaLV-1;[45] GaLV (SEATO);[420] GBr1, GBr2, GBr3.[420]
        Rodent: M-MuLV, M-MuSV(M-MuLV);[41,42] K-MuLV, K-MuSV(K-MuLV);[46] AKR MuLV;[47] F-MuLV;[48] R-MuLV;[4,49] H-MuSV;[41] RaLV (Wister-Furth);[50] RaLV (MSV-O helper);[51] RaLV (R-35);[52] HaLV[47] and HaSV.[53]
        Carnivore: G-FeLV;[47] R-FeLV;[41] T-FeLV;[53] G-FeSV;[47] R-FeSV;[53] FS-1 virus.[423]
        Ungulate: Porcine type-C particles;[54,55] Bovine type-C particles.[56,57]
      2) Avian leukosis-sarcoma virus
        MC29(ALV);[58] AMV;[42,59] B-77;[3,59] RSV(RAV-1);[42] PR-RSV;[4] SR-RSV.[3,59]
      3) Avian REV.[60,61]
      4) Reptile viper virus.[47,273]
    2. Endogenous virus
      Nonhuman primate: Baboon placenta virus (M-7).[62]
      Rodent: Mouse ecotropic viruses (N and B tropic);[63-65] Mouse xenotropic virus;[66-70,321,429] Rat endogenous virus;[71-74] Guinea pig endogenous virus;[75,76,308] Hamster endogenous virus.[77]
      Carnivore: RD114 virus;[78] CCC virus.[79]
    3. Particles isolated from human cells
      Esp-1;[80] HL23 cells;[82,425,427]
      Some human leukemic cells;[86,87,414] and normal human fibroblast strains,[234] and human carcinoma cell line.[428]
  B. Type-B virus
    734 B virus;[84] MMTV (C3H)[88] and (Paris RIII);[89] Murine milk particle (C3H)[46] and (Paris III);[42] Human milk particle.[90]
  C. MPMV[42] and MPMV-like virus
    HeLa cells;[81] Hep-2 cells;[83] HBT-3 cells;[85] AO cells.[177]
  D. Syncytium-forming viruses (primate, bovine, feline and avian).[49,91]
  E. Slow virus
    Visna virus;[49,53,92] Progressive pneumonia virus;[93] Maedi virus.[92]

II. Intracellular Particles
  A. Intracytoplasmic particles from tumor tissues
    Human leukemic cells,[6,9,13] Human breast cancer cells;[94] Human brain tumor cells;[95,96] Human *Xeroderma pigmentosum* cells;[97] Human *polycythemia vera* marrow cell line;[430] Mouse myeloma.[98]
  B. Type-C virus infected cells
    Producer
    NC-37 cells infected by SiSV-1[99] and by GaLV;[11] Mouse spleen infected by R-MuLV;[100] Chick embryo cells infected by MC-29;[101] BALB/3T3 cells infected by MuSV.[59]
    Nonproducer
    BALB/3T3 cells;[102] K-BALB/3T3 cells;[102] Rat cells transformed by RSV;[103] Chick embryo;[104] Rhesus monkey placenta.[105]
  C. Intracisternal A-particles
    Mouse neuroblastoma.[106,107]

*This table does not include all of the known virus isolates. It includes only viruses or virus-like particles reported to contain transcriptase activity.

presence of virus related components or particles both intracellularly and extracellularly from human tissues, and for synthesizing DNA probes from RNA templates. One immediate application was to work out techniques for identifying this enzyme in cells, and to distinguish it from other (cellular) DNA polymerases. Gallo and colleagues reported the first polymerase activity identified in any cell that could synthesize DNA from an RNA template.[5] This was with human leukemic blood cells. Later, this enzyme was partially purified,[6] distinguished from the known cellular DNA polymerases,[6,8,9,108] and shown to exhibit the properties of the viral polymerase[109] (see Section II.E). This finding stimulated further search for viral specific components in human tumor cells and in other biological systems. These components include RNA-dependent DNA polymerases, nucleic acids, and group-specific antigens. At present, it is clear that the virus-like DNA polymerase in virus-like intracellular particles of *some* human leukemic cells is biochemically and immunologically closely related to some known primate type-C RNA tumor viruses and some murine type-C viruses[6-9,13] (see below for detailed discussion). These findings suggest some relationship between an RNA tumor virus and the development of human leukemia. Parts of this review will place emphasis on the nature of the intracellular reverse transcriptase and its relationship to known viral reverse transcriptase and cellular DNA polymerases.

C. Nomenclature

Many names have been used in the literature for the viral DNA polymerase. This is due in part to the fact that the enzyme is able to catalyze DNA synthesis using either DNA or RNA as a template but also using an RNA primer (initiator). For example, the term, RNA-directed DNA polymerase or RNA-instructed DNA polymerase is used in order to emphasize the role and the nature of the templates since the word "dependent" does not indicate that the RNA template is transcribed. Sometimes the term "RNase sensitive DNA polymerase" is loosely used to represent RNA-dependent DNA polymerase. The term reverse transcriptase, which reflects some attachment to the classical "central dogma" of molecular biology, is the most conventional name. The proper name is the term DNA polymerase of the virus in question since it indicates the source and the basic characteristics of the enzyme without describing the mechanism of enzyme action. However, this presents a serious problem when dealing with nomenclature of a DNA polymerase believed to be viral but for which no virus has yet been identified. For this reason we prefer the term reverse transcriptase for a DNA polymerase believed to be an oncornavirus enzyme. However, the term is not used loosely. Several criteria must be met (see Section II.E). This has been the name used by most workers and agreed upon at a recent meeting on eukaryotic DNA polymerases.[110]

In theory, the catalytic action of a DNA polymerase can be divided into four types depending on whether the template and primer are DNA or RNA. These are RNA-primed RNA-directed DNA synthesis, DNA-primed RNA-directed DNA synthesis, RNA-primed DNA-directed DNA synthesis, and DNA-primed DNA-directed DNA synthesis. As shown in Table 2, both RNA-primed and RNA-directed reactions can be sensitive to pancreatic ribonuclease A. In order to distinguish among these reactions, it is necessary to perform careful product analysis. This is especially important in searching for "reverse transcriptases" using endogenous undefined primer-templates. These reactions can be distinguished by the density of the product in cesium sulphate gradients. If the size of primer molecules is relatively small compared to the size of DNA product, all RNA-directed reactions should have a DNA·RNA hybrid density (1.55 g/ml) irrespective of the nature of the primer, while the density of RNA-primed reaction products depends on the nature of the template. If the products are denatured by heat treatment, all DNA-primed reaction products have a DNA density (1.45 g/ml) while RNA-primed reaction products have a density slightly heavier than DNA. The deviation of the density from DNA depends on the size and sequence of both RNA primer and DNA product. The alkali treatment of all reaction products should release products only with the density of DNA. Another method to identify the RNA or DNA primer is to determine the transfer of α-^{32}P of deoxyribonucleoside triphosphate onto ribonucleotide or deoxyribonucleotides. If proper primer-templates are used under proper condi-

TABLE 2

Four Possible Types of DNA Synthesis Reactions with RNA or DNA as Template or Primer

Name of Reaction			Product Analysis			
			Density in Cs_2SO_4 Gradient**			
Name	Diagrammatic* presentation	RNase sensitivity	Not treated	After heat	After alkali	α-^{32}P Deoxyribonucleotide phosphate transfer to
RNA-primed RNA-directed	∿∿∿∿∿∿ ∿∿∿----	Yes	1.55	>1.45 <1.55	1.45	Ribonucleotide
DNA-primed RNA-directed***	∿∿∿∿∿∿ ———---	Yes	1.55	1.45	1.45	Deoxyribonucleotide
RNA-primed DNA-directed	———— ∿∿∿----	Yes	1.45	>1.45 <1.55	1.45	Ribonucleotide
DNA-primed DNA-directed	———— ———----	No	1.45	1.45	1.45	Deoxyribonucleotide

*∿∿: RNA; ——: template or primer DNA; --- newly synthesized DNA.
**DNA density: 1.45; DNA·RNA hybrid density: 1.55.
***Not identified in any natural system.

tions, viral DNA polymerase can catalyze all four types of reactions. Some eukaryotic cellular DNA polymerases are able to catalyze RNA-directed reactions when synthetic homopolymers are used as primer-templates but not when natural RNA is used (see below for detailed discussion), but the transcription of natural RNA is not unique to the viral DNA polymerase since *E. coli* DNA polymerase I can also transcribe some natural RNAs[111,112] including viral HMW RNA if a high concentration of the enzyme is used.[113]

With these biochemical and biological concepts in mind, in this review the names "viral DNA polymerase" and "reverse transcriptase" will be used on most occasions. Other nomenclature will be used when it is deemed necessary in the context of the discussion.

II. EXTRACELLULAR REVERSE TRANSCRIPTASE

A. Extracellular Virus and "Virus-like" Particles

From a functional point of view, an animal virus is defined as a membrane bound nucleic acid-polypeptide complex which is able to replicate itself in a permissive host and produce progeny of its kind. This definition is based on the existence of extracellular, transmissible particles. In addition to this, an RNA tumor virus is a virus which contains a HMW RNA,[114,115] which buds from cell membranes, and is often able to cause tumors in animals or transformation of cultured cells. Structurally, it consists of an outer membraneous envelope containing lipid and glycoprotein[116] and an inner core containing a central dense nucleoid bounded by an intermediate membrane.[117] The size of this particle is about 100 mμ in diameter. Reverse transcriptase is complexed with HMW RNA within the core structure.[118-121] Morphologically, extracellular oncornaviruses are classified into B-type, C-type, and many other undefined types.[117] The B-type particles have an envelope covered with tiny projections 5 to 10 mμ long and an eccentrically placed electron dense nucleoid, while the C-type particles have a relatively smooth envelope (with smaller projections) and a central electron dense nucleoid. This classification has been used to name many newly found particles. However, these morphological criteria are rather subjective and often lead to many controversies in the absence of a functional assay (Schidlovsky, personal communication). Due to the finding of a number of particles containing both reverse transcriptase and HMW RNA in a variety of biological systems (see Table 1), and due to limited availability of biological testing systems, tumorigenicity has not been a "sine qua non" of the RNA viruses containing reverse transcriptase. During this period, many names have been employed, such as leukovirus, oncornavirus, rousvirus, retra-

virus, ribodeoxyvirus, RNA→DNA polymerase containing virus, or rnadnavirus.

Each new terminology has its emphasis and its bias. Here we will call them RNA tumor viruses oncornaviruses. Since the major reason for the intense study of these viruses is the ability of at least some to cause neoplasia. In fact, those capable of transforming in vitro are among the most efficient transforming agents known. Every strain of polio virus does not cause polio yet we call them polio virus. Moreover, "RNA tumor virus" is the term in traditional use and is still the most commonly used nomenclature. For these reasons, we maintain the "older" names RNA tumor virus or oncornavirus. The term "virus" will be reserved only for those structures which have a replicating ability in a biological system. The term "particle" will be defined solely based on biochemical and physical criteria. In other words, the term "virus particle" does not imply any biological activity but it does not exclude the possibility of a biological activity.

With these definitions, the extracellular particles are classified into five categories (Table 1). They are the conventional RNA tumor viruses (type-C and B), MPMV, syncytium-forming virus, and slow viruses of sheep. Little is known regarding the reverse transcriptase of syncytium-forming viruses and slow viruses of sheep.

B. Reverse Transcriptase Activity in Crude Virus Lysates

Viral reverse transcriptase is located in the core structure of viruses or virus-like particles[118,119] and is complexed with viral nucleic acids.[120,121] In order to carry out DNA synthesis, it is necessary to make the core permeable to the exogenous nucleotides and/or primer-template or to purify the enzymes from the virions. When DNA synthesis is directed by an endogenous RNA template, the system is generally called "endogenous DNA synthesis reaction" or "endogenous reaction." The reaction is at least partially sensitive to pancreatic ribonuclease A. In general, the endogenous reactions require partial disruption of the particles, a divalent cation (magnesium or manganese), and all four deoxyribonucleoside triphosphates, and is stimulated by reducing agents, salt, and phosphatase inhibitors. The enzyme in crude lysates also can use exogenously added synthetic or natural primer-template for DNA synthesis. A confirmation of RNA-directed DNA synthesis is dependent on a biochemical analysis of the reaction product.[42]

1. Reaction Conditions

a. Permeation of the Virus Particle

Virus particles are generally made permeable by treatment with low concentrations of nonionic detergents[3,4,46] or ether.[47] Requirement for this "partial disruption", in fact, has been used as an indication of the internal location of the viral polymerase. If the virus preparation is repeatedly frozen and thawed, treatment with nonionic detergent may not be required. Ether extraction is infrequently used due to low yield of endogenous enzyme activity. This low yield of activity is not due to an inactivation of the viral enzyme, rather it is due to the destruction of the primer-template (see below for discussion). Almost all nonionic detergents are effective in partially disrupting virus particles. Among them Nonidet P-40,® Triton X-100,® and Sterox SL® are most commonly used. Each nonionic detergent has a narrow range of optimal concentrations.[46,59,122-124] High concentrations of Triton inhibit the endogenous reaction. This is not due to dissociation of the enzyme from the primer-template, since in a system using purified enzyme and viral 70S RNA, DNA synthesis is unaffected by the presence of 2% Triton X-100.[124] The more likely explanation for this inhibitory activity by nonionic detergent is that it promotes the release of nuclease activity which destroys the endogenous primer-template.[122,125] If an exogenous synthetic primer-template is added to the reaction, DNA synthesis continues even in the presence of relatively high concentrations of Triton X-100.[124] This would then suggest that the enzyme is stable in these concentrations of nonionic detergent. The optimal detergent concentrations for endogenous reactions vary with viral purity, mode of storage, age, and type of virus. Therefore, it is necessary to determine the optimal concentration for each batch of virus. The concentration of Triton X-100 used as reported in literatures ranges from 0.01% to 0.2% and of Nonidet P-40, from 0.1 to 1% (see a recent review by Green and Gerard[126]). These ranges include both the reaction with and without the addition of exogenous primer-template. In general, in a reaction with endogenous primer-template, the optimal nonionic detergent concentration is below 0.05%.

b. Primer-template

(1) Endogenous Primer-templates

Reverse transcriptase is defined by its ability to

catalyze DNA synthesis utilizing viral RNA as template. The basic observations are

1. The reaction is sensitive to RNase;[3,4]
2. The newly synthesized DNA is associated with viral HMW RNA;[120,127]
3. The purified DNA product can back hybridize to the HMW RNA;[42]
4. HMW RNA can be used as primer-template by purified reverse transcriptase.[124, 128-132]

The details of product analysis will be elaborated further in the next section. The evidence that RNA serves as a primer for reverse transcriptase to catalyze DNA synthesis using endogenous template is based on the observation that DNA products are covalently linked to an RNA.[133-136] The primer is a species of 4S RNA, which associates with HMW RNA by a hydrogen bond.[137-139]

(2) Exogenous Primer-template

The finding that some synthetic hybrids are extremely efficient primer-templates for reverse transcriptase greatly enhanced the sensitivity of the assay for reverse transcriptase. This is especially valuable for two purposes: in searching for reverse transcriptase activity in a crude preparation and for estimating and detecting extracellular viruses. However, due to the lack of specificity of the synthetic hybrids for the viral DNA polymerase, application of this finding should be performed with caution,[140] and conclusions made only after a number of criteria for reverse transcriptase have been satisfied (see Section II.E). As with other DNA polymerases, single-stranded homopolymers (DNA or RNA) are not used as templates by reverse transcriptase in the absence of primers, but additionally, double-stranded synthetic DNA or RNA homopolymers are also poorly utilized.[141-143] Hybridization of some single-stranded homoribopolymers with complementary deoxyribopolymers or oligodeoxyribonucleotides turns these synthetic duplex molecules into extremely efficient primer-templates for reverse transcriptase. Examples of these are $(dT)_m \cdot (rA)_n$, $(dT)_{oligo} \cdot (rA)_n$, $(dG)_{oligo} \cdot (rC)_n$, and $(dC)_{oligo} \cdot (rI)_n$. The efficiency of these primer-templates varies with the type of virus.[143] Reverse transcriptase asymetrically catalyzes DNA synthesis by transcribing the RNA strand of these duplex hybrid structures[141,142] and the DNA strand with a 3′-OH end serves as primer for the initiation of DNA synthesis.[144] Some synthetic double-stranded RNA such as $(rA)_m \cdot (rU)_n$ and $(rI)_m \cdot (rC)_n$ and some synthetic DNA such as $(dC)_m \cdot (dG)_n$, $(dC)_m \cdot (dI)_n$ and $(dA{=}dT)_n$ are utilized but with poor efficiency.[142,145] Native DNA and denatured DNA are also generally poor primer-templates. Native DNA activated by a partial digestion with DNase I[146] is utilized but not as well as by most cellular DNA polymerases (both eukaryotic and prokaryotic). $(dT)_n \cdot (rA)_n$ is utilized efficiently by some cellular DNA polymerase and also by reverse transcriptase. The capacity to catalyze $(dT)_n$ synthesis with crude cell extracts or with partially purified enzymes with this hybrid has been repeatedly and incorrectly used as a sole criterion for the presence of reverse transcriptase[59,147,148] (see below). Endogenous reactions can also be stimulated by the addition of DNA primer such as $(dT)_{oligo}$ and $(dG)_{oligo}$.[149,150] The reaction stimulated by $(dT)_{oligo}$ led to the finding of $(rA)_n$ sequence in viral RNA.[151-154]

While $(dT)_{oligo} \cdot (rA)_n$ is the most efficient primer-template for reverse transcriptase, $(dT)_{oligo} \cdot (dA)_n$ is virtually completely inactive as a primer-template. Therefore, the preference of a DNA polymerase to using $(dT)_{oligo} \cdot (rA)_n$ as primer-template over $(dT)_{oligo} \cdot (dA)_n$ has been suggested to be one useful (albeit not absolute) criterion for distinguishing reverse transcriptase from other DNA polymerase,[155] but since $(dT)_{oligo} \cdot (rA)_n$ is not specific for reverse transcriptase (normal cellular DNA polymerases β and γ also utilize this hybrid as primer-template,[156] especially in the presence of Mn^{++}), care must be exercised in drawing conclusions and other criteria must be satisfied. For example, the ratio of ability of DNA polymerase γ to utilize $(dT)_{oligo} \cdot (rA)_n$ over $(dT)_{oligo} \cdot (dA)_n$ is higher than 10:1 when Mn^{++} is used as divalent cation. However, this ratio is not maintained when Mg^{++} is used as divalent cation.[157] $(dG)_{oligo} \cdot (rC)_n$ has been suggested as the only specific synthetic primer-template for reverse transcriptase,[143] but this is clearly not absolute with purified enzyme systems since recently purified cellular DNA polymerase γ was shown to use $(dG)_{oligo} \cdot (rC)_n$ as primer-template.[158]

c. Divalent Cations

Like other DNA polymerases, reverse transcriptase requires either Mg^{++} or Mn^{++} for catalytic activity.[41,48] In general, the mammalian (including primate) type-C RNA tumor viruses show greater activity with Mn^{++}, while the avian viruses, the type-B virus of mice, and Mason-Pfizer monkey virus show a preference for Mg^{++}. This preference is sometimes altered, especially when the purity of the virus is not well defined and also when exogenous primer-templates are used. For example, Baltimore and Smoler reported that $(dG)_{oligo} \cdot (rC)_n$ is a better primer-template for R-MuLV reverse transcriptase with Mg^{++} and that R-MuLV reverse transcriptase prefers Mn^{++} over Mg^{++} when $(dT)_{oligo} \cdot (rA)_n$ is used as primer-template. In spite of these preferences, the use of Mn^{++} in endogenous reactions as the divalent cation yields more extensive transcription of the viral genome.[159] The optimal concentrations with Mn^{++}, are narrow (around 0.5 to 1 m*M*) while the Mg^{++} optima are broad (around 5 to 10 m*M*). The optimum concentration also depends on the type and purity of virus and on the presence of a chelating agent, e.g., EDTA. Due to the complexity of the endogenous reaction, it is not known whether the presence of both Mg^{++} and Mn^{++} have a synergistic effect on the DNA polymerase activity. However, when an enzyme prefers Mn^{++}, the presence of Mg^{++} at a concentration higher than 1 m*M* is usually inhibitory. Other cations have also been studied such as Cu^{++}, Hg^{++}, Co^{++}, Zn^{++}, Cd^{++}, Ni^{++}, and Ag^{+}.[160] None of them are helpful for DNA synthesis in the endogenous reaction, but zinc ion has been reported to be associated with the native polymerase.[161,162]

d. Deoxyribonucleoside Triphosphates

For a complete reaction, all four dexoyribonucleoside triphosphates are required: dATP, dGTP, dCTP, and dTTP. Omission of one or more deoxyribonucleoside triphosphate results in reduction in DNA synthesis. The magnitude of the reduction depends on the purity of the virus and sometimes on nucleoside triphosphate. In general, omission of one deoxyribonucleotide results in about a 70% reduction and omission of more than two deoxyribonucleotides a 90 to 100% reduction. The absolute requirement of four nucleoside triphosphates indicates that reverse transcriptase is able to transcribe heteropolymeric portions of viral RNA, and that its activity is not a form of terminal addition. The concentration of nucleoside triphosphate used in the endogenous reaction by different laboratories varies greatly. It ranges from 0.05 m*M* to 8 m*M* for unlabeled dNTP and 0.06 μM to 0.1 m*M* for labeled dNTP (for a detailed summary, see Reference 126). No systematic study of the Km of each dNTP in an endogenous reaction has been reported yet. The effect of different amounts of the dNTP (especially the ratio of unlabeled dNTP to labeled dNTP) on the kinetics of DNA synthesis also remains to be studied. Recently, Rothenberg and her colleagues observed that the high concentration of dNTP (as high as 5 m*M*) is essential in obtaining endogenous DNA product with higher molecular weight (personal communication). Nucleoside diphosphates and monophosphates are not substrates for the enzyme. Sometimes ATP and CTP have been included in the reaction mixture and reported to stimulate DNA synthesis, although not incorporated into DNA.[163] One possible interpretation of this result is that AMP and CMP are incorporated into the CCA termini of 4S primer molecules[164] since a tRNA nucleotidyltransferase is found both in mammalian[165,166] and avian type-C RNA tumor viruses.[167]

RNA of RNA tumor viruses contains a track of $(rA)_n$.[151-153] The $(rA)_n$ sequence is not usually transcribed in the endogenous reaction.[168,169] However, occasionally and for unknown reasons the $(rA)_n$ sequence is extensively transcribed (Reitz, M. S., unpublished observation). Hence, it is advisable not to use dTTP as the radioactively labeled dNTP for verifying transcription of the RNA heteropolymeric regions.

e. Monovalent Cations and Reducing Agents

Either KCl or NaCl stimulates DNA synthesis with an optimal concentration around 0.05 to 0.1 *M*. The optimal concentration has a broad distribution and varies depending on the buffer used in the virus suspension, the purity of the virus preparation, and the type of virus. A salt concentration higher than 0.2 *M* is generally inhibitory. There appears to be little difference between KCl and

NaCl. The concentrations reported in the literatures range from 0 to 0.12 *M*.[126]

The most commonly used reducing agents are DTT and β-mercaptoethanol. Initially, DDT was reported to be essential for DNA synthesis.[4] However, this is debatable when the purity of the virus is not well defined. The concentrations reported range from 1 m*M* to 300 m*M*.

f. pH and Temperature Optima

The reported values of pH optima range from 7.5 to 8.3. The temperature optima of the endogenous reaction using endogenous primer-template seems to reflect the temperature of the host cells. For mammalian virus the temperature optima is about 37°C[4,41] and for avian virus 40°C.[3,59,119] However, when exogenous primer-templates are used with crude enzyme preparations, the optimal temperature varies with the thermal stability of the primer-template.[170] For example, when $(dT)_{oligo}\cdot(rA)_n$ or $(dT)_m\cdot(rA)_n$ is used, the optimal temperature is 25°C to 30°C.

g. Ribonuclease Sensitivity

The original definition of reverse transcriptase is based on its sensitivity to ribonuclease A in an endogenous reaction[3,4] since the template is single-stranded RNA. The proper method to test RNase sensitivity is to use a relatively low ribonuclease concentration (10 to 20 μg/ml) and relatively high salt concentration (0.1 to 0.2 *M* KCl or NaCl). If the concentration of salt is too low and the nuclease concentration too high, degradation of RNA in a hybrid form may occur. If this is the case, it will be difficult to determine whether the RNase sensitivity is due to degradation of a requisite primer RNA in hybrid form or due to degradation of a single-stranded RNA template. As shown in Table 2, this is especially important when one deals with RNA-primed DNA-directed DNA synthesis reaction. An example of such reaction is the cytoplasmic particulate fraction isolated from peripheral blood lymphocytes stimulated by phytohemagglutinin (PHA).[171,172] In this case, sensitivity of an endogenous reaction to RNase could lead to an erroneous conclusion that it was RNA-directed.

2. Analysis of Endogenous Reaction Products

Since the most important characteristic of reverse transcriptase is its ability to catalyze the synthesis of DNA directed by natural RNA template, the products of an endogenous reaction should be carefully analyzed and meet the following three criteria:

a. The reaction products are DNA.

The fact that the reaction requires all four deoxyribonucleoside triphosphates suggests that the product is DNA. More definite proof is obtained by showing that the product is sensitive to DNase, bands at a density of 1.45 g/ml in a cesium sulphate equilibrium gradient, and is resistant to RNase and alkali. Generally, the size of the product is small (about 4 to 6S), even though the template is large. Sometimes the product is so small that it is not acid precipitable upon dissociation from the primer-template.[172] The presence of nuclease, predegradation of template, and non-specific attachment of the radioactively labeled nucleotides on the filters are among the possible interpretations of these findings. This might account for some loss of activity upon heat denaturation or alkaline treatment.

b. The DNA products are associated with primer-template RNA.

A simple method to demonstrate that the DNA product is associated with HMW RNA is (1) to band the nucleic acids extracted from a short-term reaction (no more than 5 min) in a cesium sulphate equilibrium density gradient and show that the labeled DNA moves as RNA (since the RNA is large and the DNA small); (2) to show that the DNA product cosediments with HMW RNA in a glycerol velocity gradient. This method was named the "simultaneous detection" technique by Schlom and Spiegelman[127] (for simultaneous detection of "viral" RNA and reverse transcriptase) and is frequently used as an initial indication of the existence of virus-related particles, especially those isolated from cellular cytoplasm (see below for more detailed discussion). Although HMW RNA is unique to viral RNA, there are many other reasons that could explain the presence of radioactivity in the HMW region. One should remember that a positive result from this method may be used as an indication but not as a conclusion conclusion for the existence of a complex of the viral polymerase and nucleic acids. A firmer conclusion can be obtained if one isolates DNA from the HMW region and hybridizes this DNA back to the purified HMW RNA or by more thorough product analysis (see below).

Another method to show that DNA products

are associated with RNA is to measure the density of the DNA product before and after dissociation from the primer-template. This can be measured in a cesium sulphate equilibrium gradient in which DNA, RNA·DNA hybrids, and RNA band at 1.45, 1.55, and 1.65 g/ml, respectively.[42,173] With this method, DNA products purified directly from the reaction mixture should band at the hybrid region if the size of the DNA and RNA are comparable or at the RNA region if the size of the DNA is relatively smaller than that of the RNA. If the products are denatured by heating or by treatment with formaldehyde or with DMSO, the products should then band at either the DNA or RNA region depending on the nature of primer and/or on the size of the RNA primer. If the products are treated with alkali or RNase, they should band at the DNA density on a cesium sulphate gradient.

At early time periods of DNA synthesis, the products are associated with template RNA by hydrogen bonds and with primer by a covalent bond.[173-175] The DNA product naturally moves with RNA. However, when DNA synthesis is prolonged, in most cases the DNA products are either released as single-stranded DNA or converted to double-stranded DNA.[174-177] This conversion can be inhibited in the presence of a high concentration of actinomycin D (50 to 100 μg/ml).[174-178] Probably reverse transcriptase, ribonuclease H, and other specific nucleases are required for this conversion.

c. Reassociation of the DNA product to the non-$(rA)_n$ portion of the RNA template.

In order to obtain conclusive evidence that RNA is the template of the DNA product, it is necessary to demonstrate that the DNA product is homologous to the heteropolymeric portion of the RNA template. There are two reasons for this. First is to rule out the possibility of nonspecific association of the DNA product to the RNA template; second is to prove that the product is not $(dT)_n$. As discussed above, the RNA of these viruses contain a track of $(rA)_n$.[151-154] The $(rA)_n$ sequence is located at 3′-OH end of the RNA[179] and is not generally transcribed by reverse transcriptase from avian[168] or mammalian viruses.[169] Under certain circumstances the $(rA)_n$ sequence is copied, for example, when the DNA synthesis is carried out in the presence of $(dT)_{oligo}$. The simplest method to show that the entire product is not just $(dT)_n$ and hence to indicate that heteropolymeric regions are at least in part transcribed, is to use labelled dNTP's other than dTTP. It is still possible that the DNA product is linked to $(dT)_n$. Molecular hybridization is used to show that the sequences maintained in the DNA product and viral RNA are complementary. The quantity of hybrid can be determined by banding the hybrid in a cesium sulphate density gradient[42,173] in which the DNA bands in the RNA region, by resistance of the hybrid to S1 nuclease,[180] and by retention of hybrid to a cellulose filter to which the RNA is covalently bound.[181]

3. Some Properties of the DNA Product

a. Extent of Transcription of 70S RNA

Generally, the DNA products synthesized *in vitro* do not uniformly represent the viral 70S RNA genome. Duesberg and Canaani reported that RSV RNA was completely copied in an endogenous reaction but 85% of the DNA product was complementary to only 15% of the viral genome.[182] The experiment was performed by measuring the degree of protection of ^{32}P-RNA to RNase after hybridizing excess amounts of DNA product to viral RNA. The ratio of amount of DNA to RNA by weight was more than 100:1. Similar findings were reported by Bishop et al. with RSV[183] and Gelb et al. with mammalian viruses[184] using reassociation kinetics.[185] With the protection experiment, the products from many mammalian type-C viruses have been found to represent more uniform copies of the viral genome than those of avian viruses. For example, Benveniste et al. reported that 70% of the viral genome was protected from S1 nuclease digestion by hybridizing with only a threefold excess of DNA product to viral RNA.[186] It has been further claimed that in R-MuLV, the whole 70S RNA was uniformly transcribed.[187] Two conditions have been reported to favor the extent of copying the HMW RNA. One is Mn^{++} ion,[158,159] and the other is the presence of actinomycin D in the reaction.[188] Recently DNA products have been used extensively as probes to study the mechanism of viral replication to clarify the origin and evolution of these viruses and to search for viral derived nucleic acids in viral transformed nonproducing cells. It is important to obtain methods which allow for synthesis of DNA products which uniformly represent the viral genome or which at least indicate the fraction of the genome represented.

b. Size of the DNA Products

The size of the DNA products with the endogenous primer-template is about 4 to 10S.[59,174,176,189] This is equivalent to only about 100 to 150 nucleotides. Prolonged incubation does not increase the size of the DNA product, but most of the DNA is converted to double-stranded DNA of similar size.[161,174-178] Sometimes the products are so small that, although they are acid precipitable when associated with primer-template, they become acid-soluble upon dissociation from primer-template.[172] The addition of a phosphatase inhibitor such as NaF to the reaction mixture may prevent the synthesis of these small products. Recently, Guntaka et al. reported that the final form of the DNA product of RSV upon infection of duck cells is a double-stranded helical circle with an estimated molecular weight of six million,[190] and similar findings were made by Gianni et al. in mouse cells infected by murine leukemia virus.[191] Therefore, there is a clear discrepancy between the in vitro endogenous reaction and intracellular proviral DNA synthesis. Lack of cellular factors or an inappropriate microenvironment might be reasons for the endogenous in vitro reaction. Apparently, high concentrations of dNTP (as high as 5m*M*)[43,7] and conditions unfavorable for nuclease activity, such as optimal nonionic detergent concentration, tend to enhance the rate of DNA synthesis and result in larger sized DNA products. In some cases some products with a molecular weight of 2.5 to 3×10^6 daltons were obtained; however, these high molecular weight products are only a small portion of the total product. When exogenous synthetic hybrids are used as primer-templates, the size of the product is larger than the RNA strand of the hybrid, but when activated natural DNA is used, the size of product is equivalent to the size of the gaps digested by DNase.[192]

c. Covalent Linkage of DNA Product to RNA Primer

Among 16 possible linkages between the DNA product and the RNA primer, Verma et al. found that only the rA-dA linkage was present in the DNA product of the endogenous reaction from AMV disrupted by NP40.[134] The same finding was obtained from RSV,[192] B77 virus,[193] and R-MuLV[194] when the virions were disrupted by NP40. However, when AMV was disrupted with ether, Flügel et al. found that there are two types of RNA-DNA linkages: a major bond, rU-dC and a minor bond, rA-dA.[135] The ether-disrupted B77 virus and R-MuLV also had two types of linkages, rC-dC, the major bond and again rA-dA, the minor bond.[193] To further elucidate the effect of disruption of the virions on the RNA-DNA linkage, Flügel et al. showed that when B77 virus was treated with both NP40 and ether, only the rA-dA linkage was found.[135] Another factor which affects the RNA-DNA linkage is the age of the virus. Flügel et al. observed that in the endogenous reaction of R-MuLV obtained after only 5 min pulse with labeled RNA precursor, there were mainly rC-dC linkages with ether disrupted virus. However, with virus harvested after 24 hr and disrupted by ether, there were both rC-dC and rA-dA linkages.

Clarification of these discrepancies and a final interpretation of the findings will depend on a detailed understanding of the sequence of the primer structure, and this has recently been achieved by Dahlberg and his associates. They have obtained the complete sequence of the primer 4S RNA (a species of tryptophan tRNA) from Rous sarcoma virus. The sequence of 3′-OH end was shown to be -UCACCAOH.[195] These results, of course, support the rA-dA linkage. It is still possible that other primers are used in the presence of some detergents. It will be important to determine if the primer used in vivo is the same as the tryptophan tRNA primer discovered in vitro.

d. Base Composition of the DNA Products

Several lines of information indicate that DNA products of the endogenous reaction are heteropolymers:

1. The DNA products are not $(dT)_n$;[168]
2. The DNA products are complementary to heteropolymeric portions of viral HMW RNA both in avian[42,136] and mammalian[42,173] virus systems;
3. Nearest-neighbor analysis of the DNA product synthesized with one ^{32}P-labeled deoxyribonucleoside triphosphate and three unlabeled triphosphates showed that the DNA products are heteropolymers composed of all four nucleotides.[42,150]

TABLE 3

Summary of Procedures for Purifying Viral Reverse Transcriptase from Extracellular Particles

Reference	Virus	Solubilization method*	Fractionation procedures: Procedures** and Molarity of Enzyme Eluation	Buffer***	pH	Molecular weight† (× 10^{-3} daltons)	Purification	Presence of RNase H
			I. Conventional column procedures					
Kacian et al.[128]	AMV	7% NP40 0.8 *M* KCT 0.7% Na deoxycholate (95%)	DEAE-Cell (0.3G)	KP (10)	7.2			
			CM-Seph.® C-50	KP (10)	8.0		40	
			PC (0.2G)	KP (10)	8.0			
			GG (0.2)	KP (10)	8.0	110 (V.S.) 6S		NR††
			Seph. G200 (0.2)	KP (10)	8.0	110 (SDS) 69 (SDS)		
			HA (0.25G)	KP (10)	7.2			
			DNA Cell (0.25G)	KP (10)	8.0			
Baltimore and Smoler[224]	AMV	1% NP40	A-25 DEAE-Seph. (0.1G)	Tris (25)	7.9			+
			PC (0.3G)	Tris (25)	7.9	NR	50	+
			GG (0.2)	Tris	7.5			+
			GG (0.5	Tris	7.5			+
Grandgenett et al.[227]	AMV	2% NP40 (99%)	DEAE-Cell (0.09G)	KP (10)	8.0			+
			PC PI (0.11G)	KP (10)	8.0			+
			PII (0.22G)			NR		+
			GG PI (0.35)	Tris	8.0	90 (V.S.)		+
			PII (0.35)	Tris	8.0	160 (V.S.)		+
						α 65 (SDS)	NR	+
						β 105 (SDS)		NR

*Number in parenthesis represents the percentage of enzyme recovered in supernatant after solubilization.
**PC: Phosphocellulose; GG: glycerol gradient; HA: hydroxylapatite; Seph: Sephadex; ASP: amonium sulfate precipitation; PA: Peak A; PB: Peak B; PI: Peak I; PII: Peak II; IgG: immunoglobulin G. The information in parenthesis represents the molarity of salt at which enzymes are purified in a velocity sedimentation and gel filtration procedure or the molarity of salt at which enzymes are eluted from ionic exchange chromatography. Some abbreviations are as follows: G: eluted by salt gradient; B: eluted stepwise by a salt buffer, and others in case of velocity sedimentation and gel filtration.
***KP: Potassium phosphate buffer; NaP: sodium phosphate buffer. The number in parenthesis represents the percentage of glycerol in the buffer.
†V.S.: Molecular weight estimated by velocity sedimentation in glycerol gradient or in sucrose gradient; SDS: molecular weight determined by a SDS polyacrylamide gel disc electrophoresis; GF: molecular weight estimated by gel filtration.
††NR: Not reported.

TABLE 3 (continued)

Summary of Procedures for Purifying Viral Reverse Transcriptase from Extracellular Particles

Reference	Virus	Solubilization method*	Fractionation procedures: Procedures** and Molarity of Enzyme Elution	Buffer***	pH	Molecular weight† (× 10^{-3} daltons)	Purification	Presence of RNase H
Mölling et al.[214]	AMV	0.5% NP40	Sucrose gradient NR	NR		NR	NR	+
			DEAE-Seph. (0.075G)	Tris (5)	7.5			+
Waters and Yang[199]	AMV	0.8% NP40	GG (0.5)	Tris	8.0	GS (V.S.)		
		0.5 *M* KCl	HA (0.04G)	KP (15)	7.2			
		Frozen and thaw	PI (0.05G)	Tris (15)	8.0		50–110	
			PII (0.25G)					+
	R-MuLV		GG (0.5)	Tris	8.0	4S (V.S.)		
			HA (0.025G)	KP (15)	7.2			
			PC (0.25G)	Tris (15)	8.0		100–150	+
Hurwitz and Leis[145]	AMV	0.4% Triton® X-100	ASP (40%)	Tris HCl	8.0			
		0.1 M NaCl (90–95%)	PC PI (0.05G)	Tris (10)	8.4			
			PII (0.15G)					
			GG PII (0.05G)	Tris	7.9	160 (V.S.)	80	NR
	R-MuLV	0.4 Triton X-100	ASP (40%)	Tris	8.0			
		0.1 *M* KCl	PC (0.22G)	Tris (5)	8.0			
			GG (0.05)	Tris	7.9	90 (V.S.)	75 < > 75	NR
Duesberg et al.[272]	PR-RSV/C	0.5% Triton X-100 0.02 *M* KCl	DEAE Cell (0.15–0.2G)	Tris (5)	7.4	170 (SDS) 8S	40	
	SR-RSV/A	(75–80%)	RNase treatment			110 (SDS) 6S		NR
Faras et al.[123]	SR-RSV	1% NP40 (75%)	DEAE-Cell (0.4B)	Tris (30)	7.2 (30%)			
			PC PA (0.2G)	NaP	6.8			
			PB (0.4G)					
			Seph. G100 PA (0.4)	NaP (30)	6.8	96 (GF)		NR
			PB (0.4)	NaP (30)	6.8	105 (GF)		
			GG (20–40%) PA (0.4)	NaP	6.8	5.3S (V.S.)	940	
			PB (0.4)	NaP	6.8	6.2S (V.S.)		

TABLE 3 (continued)

Summary of Procedures for Purifying Viral Reverse Transcriptase from Extracellular Particles

Reference	Virus	Solubilization method*	Fractionation procedures: Procedures** and Molarity of Enzyme Elution	Buffer***	pH	Molecular weight† ($\times 10^{-3}$ daltons)	Purification	Presence of RNase H
Wang and Duesberg[270]	PR-RSV-B PR-RSV-C K-MuSV (K-MuLV) M-MuLV M-MuSV (M-MuLV) R-MuLV	0.5% Triton X-100	AS (45%) Seph. G200 (0.4) GG (0.1)	NaP (30) Tris	6.2 8.1	160 (V.S.) RSV .70 (V.S.) [MuLV(MuSV)]	NR	±
Ross et. al.[421]	R-MuLV	1% Triton 0.5 *M* KCl 1 *M* Urea 20% Glycerol	PEG-Dextran Seph. G100 (0.3) PC (0.4G) GG (0.15)	Tris (20) Imidazole (20) Tris	7.8 6.5 7.8	70 (GF) 70 (V.S.)	100	NR
Wu et al.[257]	R-MuLV K-MuSV (K-MuLV)	1% Triton 0.8 *M* KCl 20% Glycerol	PC (0.4G) DEAE-Cell (0.2G) GG (0.3)	Tris (20) Tris (20) Tris	7.9 (20) 7.9 (20) 7.9	70 (V.S.)	160	+ + +
Wu et al.[201]		1% Triton 0.8 *M* KCl 20% Glycerol	DEAE-Cell PI (0.05G) PII (0.14G) PC PI (0.2G) PII (0.2G) GG PI (0) (0.5) PI (0) (0.5)	Tris (20) Tris (20) Tris Tris	7.9 (20) 7.9 (20) 7.9 7.9	70 (V.S.) 70 (V.S.) 150 (V.S.) 70 (V.S.)		+ + + + + + + +
Grandgenett et al.[256]	R-MuLV	0.2% Brij. 58® 0.1 *M* NaCl	Seph. G200 (0.5) DEAE-Seph. A-50 (0.2G) Sephgrose® 6B (0.1) (0.5)	Bicine-NaOH KP (10) KP (10)	7.5 7.5 7.5	480 (G.F.) 70 (G.F.) 135 (G.F.)	150	NR

TABLE 3 (continued)

Summary of Procedures for Purifying Viral Reverse Transcriptase from Extracellular Particles

Reference	Virus	Solubilization method*	Fractionation procedures: Procedures** and Molarity of Enzyme Eluation	Buffer***	pH	Molecular weight† (× 10^{-3} daltons)	Purification	Presence of RNase H
Weimann et al.[48]	F-MuLV	Triton X-100 Sonication	ASP (20–50%)					
			DEAE-Cell (0.15G)	Tris (20)	8.0			+
			PC (0.3G)	Tris (20)	8.0			+
			GG (0.3)	Tris	8.0	123 (V.S.)		
						50–67 (SDS)		+
Mölling et al.[131]	F-MuLV	7% NP40	DEAE-Cell (0.3B)	KP (20)	7.2			+
		0.8 *M* KCl	CM Seph.-50 (0.3B)	KP (20)	8.0			+
			GG (0.3)	KP	8.0	84 (V.S.) (SDS)		
						45–56		+
			DEAE-Cell (0.07G)	KP (20)	7.2			
			PC (0.2G)	KP (20)	7.2			
Howk et al.[208]	RIII Milk	2% Triton X-100	PC A (0.18G)	Imidazole (20)	6.8 (20)			
			B (0.3G)					
			Seph. G100 (0.3)	Tris (20)	7.5	NR	NR	NR
	NIH Swiss BALB Milk MPVM GaLV	2% Triton X-100	PC (0.3G)	Imidazole (20)	6.8			
			Seph. G150 (0.3)					
Abrell and Gallo[196]	M-PMV	0.25% Triton X-100						+
		0.5 *M* KCl						+
	SSV-1	0.25% Triton X-100	DEAE-Cell (0.05G)	Tris (5)	7.9			+
			Sucrose Gradient (0.35)			110 (V.S.)	800	+
	R-MuLV	0.5 *M* KCl	PC (0.15G)	Tris (20)	7.9 (20)	70 (V.S.)		+
Twardzik[273]	Viper type-C	Detergent	PC (0.2G)	KP (20)				
			GG (0.5)	Tris	7.8	109 (V.S.)	NR	NR

TABLE 3 (continued)

Summary of Procedures for Purifying Viral Reverse Transcriptase from Extracellular Particles

Reference	Virus	Solubilization method*	Fractionation procedures: Procedures** and Molarity of Enzyme Eluation	Buffer***	pH	Molecular weight† (× 10^{-3} daltons)	Purification	Presence of RNase H
			II. Affinity chromatographical procedures					
Livingston et al.[203]	SR-RSV	1% Triton	Seph. G100 (0.3)	Tris (20)	7.8			
	G-FeSV	1 *M* Urea	IgG Sepharose (0.2 *M* NH_4OH)	Tris	7.8			
	R-MuLV	0.5 *M* KCl 25% Glycerol	0.3 *M* KCl 1% BSA (pH 10.6)					
Livingston et al.[204]	RAV-1 SR-RSV AMV		Seph. G100 (0.3)	Tris (20)	7.8			
			IgG Sepharose (0.05 *M* NH_4OH, 1% BSA, 25% Glycerol [pH 10.2–10.3])	Tris	7.8			
Gerwin and Milstein[202]	R-MuLV	1% Triton	oligo(dT)-Cell (0.25G)	KP (20)	7.1			
	RD114 virus	0.5 *M* KCl	PC (0.3G)	Imidazole (20)	6.5			
			Seph. G100 (0.1)	Tris (20)	7.8	50 (G.F.)		
Marcus et al.[198]	AMV	1% NP40	Poly(rC)-Agarose (0.2G)	KP (10)	8.0	68 (SDS)	40	NR
		0.4 *M* Na deoxycholate 0.4 *M* KCl				105 (SDS)		
		20% Glycerol	GG (0.4)	KP	8.0	6.6S (V.S.)		
Chirikjian et al.[239]	AMV MMTV	NR	Pyran-Sepharose (0.38G); (0.22G)	KP (20)	7.2	NR	NR	NR
	R-MuLV		(0.25G)					
	RSV		(0.4G)					
	FeLV		(0.3G)					

C. Purified Reverse Transcriptase Activity

1. Purification

The purification of reverse transcriptase from extracellular virus particles is relatively simple compared to that of the purification of an enzyme from whole cells, since there are few species of protein and nucleic acids in the virions, and no other polymerase. The best purifications reported are about 1,000-fold. These are reverse transcriptase of Rous sarcoma virus[123] and of MPMV.[196] However, due to a difficulty in obtaining large quantities of some purified viruses, not many viral reverse transcriptases have been studied critically from a biochemical point of view. For example, in most cases there is not enough protein to determine its concentration and therefore the purity or specific activity cannot really be determined. Table 3 shows a summary of the procedures and results of the purification of viral reverse transcriptase reported to date (for a recent review, see Reference 197). The purification procedures can basically be divided into two phases. One is solubilization of the enzyme and the other is the fractionation of the enzyme molecules from other components.

a. Solubilization

Reverse transcriptase of RNA tumor viruses can be solubilized from virions by a treatment with nonionic detergents such as Nonidet 40 or Triton X-100. As shown in Table 3, the concentration of each nonionic detergent used varies with the type of virus, with other components in the solubilization buffer, and with laboratory preferences. The salt concentration also varies greatly in different reports. High salt concentration favors dissociation of the enzyme from other viral components including nucleic acids.[128] To enhance the solubilization of the enzyme, 1 *M* urea[123] or 0.7% deoxycholate[128,198] is sometimes added to the solubilization buffer or the procedures such as freezing and thawing[199] and sonication[48,196,200] are used. There is no reported evidence that these additional reagents and procedures are necessary. Without exception, glycerol (concentration ranging from 5 to 30%) and a sulfhydryl agent (1 m*M* to 10 m*M*) have been included in all solubilization buffers. Solubilization is usually done at 0° to 4°C for ½ to 1 hr, although some prefer to incubate at 37°C. The initial step to separate the solubilized enzyme from unsolubilized and high molecular weight components (such as nucleic acids) is ultracentrifugation at about 50,000 xg (varying from 16,000 to 100,000 xg) for 30 to 60 min. With this procedure, about 75 to 99% of the enzyme is recovered in the supernatant (see Table 3).

b. Fractionation

(1) Conventional Fractionational Procedures

Ammonium sulphate precipitation is rarely used for fractionation of reverse transcriptase, but it is used occasionally for concentrating the enzyme. The commonly used procedures for fractionation involve ionic exchange chromatography, gel filtration, and velocity sedimentation. The combination of some of these procedures has been shown to be very efficient for enzyme purification.

(a) Ion Exchange Chromatography

Since reverse transcriptase is a DNA-binding protein and most of the other viral components are not, processing disrupted viruses through a phosphocellulose column is usually sufficient to purify reverse transcriptase about 50- to 100-fold. This degree of purification is useful for many practical purposes. However, in the authors' experience, often some enzyme remains in the flow-through due to adherence to nucleic acids. To overcome this problem, nucleic acids should first be removed by DEAE cellulose column chromatography or a similar simple step. This process removes most of the loose binding nucleic acids, but some tight binding nucleic acids (such as primer) are still bound to the eluted enzyme. Almost all of the DEAE eluted enzyme can then be adsorbed onto phosphocellulose. The PEG-dextran procedure also has been used to remove nucleic acids.[421] DEAE sephadex, CM sephadex, hydroxyapatite, and DNA cellulose are other types of ion exchange columns that have been used for polymerase purification. When small amounts of virus are used for enzyme purification, the presence of low concentrations of nonionic detergent and reducing agent enhance enzyme recovery.[196] The salt molarity used in elution of enzyme from these columns varies with many factors, particularly with pH and components of the buffer and also with the concentration of nonionic detergents. Therefore, comparison of values from different reports is not

meaningful. Most of the enzymes purified through ion exchange chromatography still contain *some other viral components*, such as group specific proteins (Gallo and Wu, unpublished observation) and RNase H (see Table 3).

(b) Gel Filtration and Velocity Sedimentation

Factors other than the charge of the enzyme are used in the fractionation of reverse transcriptase, for example, size and shape. Thus, gel filtration with Sephadex® and velocity sedimentation with glycerol or sucrose density gradients are frequently utilized. Both Sephadex G-100 and G-200 are used. For velocity sedimentation, a 10 to 30% glycerol gradient is common. Besides being a step in purification, this procedure can be used to obtain a sedimentation size estimate of the enzyme. Since reverse transcriptase enzyme aggregates in low salt,[11,201] it is advisable to purify the enzyme through these steps in a high salt buffer for estimates of enzyme size.

In view of the ease of purification of reverse transcriptase from virus by conventional purification procedures, affinity chromatography is not particularly useful. However, a development of highly specific affinity chromatography would be extremely useful in purifying reverse transcriptase from cells.

(2) Affinity Chromatography

(a) $(dT)_{oligo}$-Cellulose Chromatography

The use of $(dT)_{oligo}$-cellulose chromatography was based on the observation that $(dT)_{oligo} \cdot (rA)_n$ is a very efficient primer-template for reverse transcriptase. Gerwin et al. reported that cellular DNA polymerases are not adsorbed by $(dT)_{oligo}$-cellulose, and that this type of chromatography was unique for viral reverse transcriptase.[202] Since $(dT)_{oligo} \cdot (rA)_n$ is not absolutely specific for reverse transcriptase, unique adsorption of reverse transcriptase was perplexing. Indeed, other investigators have found that it is sensitive to nucleases and that some cellular DNA polymerases do adsorb to this column (Reference 424 and M. Sarngadharan, unpublished observations).

(b) $(rC)_n$-Agarose Chromatography

Among all of the synthetic primer-templates, $(dG)_{oligo} \cdot (rC)_n$ is probably the most specific for reverse transcriptase. Marcus et al. reported that with $(rC)_n$-agarose affinity column, a 40-fold purification was achieved.[198] The eluted enzyme still contained RNase H activity and p27 protein. *E. coli* DNA polymerase was also adsorbed to the column but eluted at a lower salt concentration compared to reverse transcriptase. Recently, Carl Saxinger at the National Cancer Institute has developed a variety of polynucleotide affinity chromatography columns with a cellulose matrix. None of them have a unique specificity to reverse transcriptase but a combination of various types of polynucleotide cellulose columns is potentially a very useful method for separation of reverse transcriptase from other cellular enzymes (Saxinger, personal communication).

(c) Pyran-Sepharose Chromatography

Chirikjian et al. reported that pyran-Sepharose is an effective affinity column for the purification of reverse transcriptase from several RNA tumor viruses.[239] Analysis of iodinated proteins by SDS gel electrophoresis revealed that the reverse transcriptase from AMV could be purified nearly to homogeneity by a single passage through the column. Since pyran-Sepharose is totally resistant to nuclease digestion and is also stable at 4°C for several months, this could be a useful procedure to isolate reverse transcriptase from crude cell lysates. In general, viral reverse transcriptases have a higher affinity to pyran-Sepharose than prokaryotic DNA polymerases. Since the affinity of eukaryotic cellular DNA polymerase to this column was not shown, the application of this procedure to crude cell lysate system remains to be evaluated.

(d) Immunoadsorbant Chromatography

In immunoadsorbant chromatography, monospecific IgG to specific reverse transcriptase is linked to Sepharose.® This then can be used to purify reverse transcriptases both from virus and infected cells.[203,204] IgG affinity chromatography has been developed both in avian[204] and in mammalian systems.[203] One problem with this method is the difficulty of obtaining sufficient specific IgG for an efficient performance.

2. Reaction Conditions

Purified enzymes, of course, require exogenous primer-templates for their catalytic activity. The optimal conditions for DNA synthesis vary with the primer-template used and also with the type of virus.

a. DNA synthesis Using Synthetic Primer-template

Most of the synthetic primer-templates that stimulate crude enzyme from disrupted virions can

TABLE 4

Some Biochemical Properties of Viral Reverse Transcriptase

Properties	Type C		Type B	MPMV
	Avian	Mammalian		
Preferred divalent cations*				
Activated DNA $(dA\text{-}dT)_n$	$Mg^{++} >> Mn^{++}$	$Mg^{++} > Mn^{++}$	NR**	NR
$(dT)_m \cdot (rA)_n$	$Mg^{++} = Mn^{++}$	$Mn^{++} > Mg^{++}$	NR	$Mg^{++} > Mn^{++}$
$(rA)_m (rU)_n$	$Mn^{++} > Mg^{++}$	$Mn^{++} > Mg^{++}$	NR	NR
(dT)oligo · $(rA)_n$	$Mg^{++} >> Mn^{++}$	$Mn^{++} > Mg^{++}$	$Mg^{++} > Mn^{++}$	$Mg^{++} > Mn^{++}$
(dG)oligo · $(rC)_n$	$Mg^{++} >> Mn^{++}$	$Mg^{++} = Mn^{++}$	$Mg^{++} > Mn^{++}$	$Mg^{++} > Mn^{++}$
70S RNA	$Mg^{++} > Mn^{++}$	$Mn^{++} = Mg^{++}$	NR	$Mn^{++} > Mg^{++}$
70S RNA + (dT)oligo	$Mg^{++} > Mn^{++}$	$Mg^{++} = Mn^{++}$	NR	NR
Molecular weight	α 70,000 β110,000	70,000	110,000	110,000
Separation of RNase H	Not separable	Separable	NR	NR

*The optimal concentration of Mg^{++} is around 5-10 m*M* and that of Mn^{++} is around 0.5-1 m*M*. The comparison is made based on their activity at optimal concentrations. The relative preference for divalent cations differs in many reports. These represent the most frequently used conditions recognized by the authors.

**NR: Not reported.

be used as primer-templates for purified enzymes. Among them $(dT)_{oligo}\cdot(rA)_n$, $(dT)_m\cdot(rA)_n$, $(dG)_{oligo}\cdot(rC)_n$, $(dA\text{-}dT)_n$ are the most efficient.[123,128,196,199,200] Purified reverse transcriptase efficiently uses the hybrid $(dT)_{oligo}\cdot(rA)_n$ as primer-template, but not its DNA counterpart $(dT)_{oligo}\cdot(dA)_n$.[124,200,205] DNA polymerase γ[99,156,158,281] and β of mammalian and chicken cells[157,206] and bacterial DNA polymerase[205] are able to use $(dT)_{oligo}\cdot(rA)_n$ as primer-template, but they also use $(dT)_{oligo}\cdot(dA)_n$ with fair to high efficiency. Therefore, the lack of utilization of $(dT)_{oligo}\cdot(dA)_n$ by a viral enzyme provides a criterion to distinguish between cellular and purified viral DNA polymerases. Since there has been no definite demonstration that cellular DNA polymerases (except DNA polymerase γ) can use $(dG)_{oligo}\cdot(rC)_n$ as primer-template, this is probably a better synthetic primer-template for reverse transcriptase under optimal conditions. $(dT)_n\cdot(rA)_m$ is commonly employed to screen for reverse transcriptase and for DNA polymerase γ due to its high efficiency as primer-template.

Recently, Gerard et al. found that a hybrid structure between oligodeoxyribguanoylate and poly(2′-O-methylcytidylate) $[(dG)_{oligo}\cdot(Cm)_n]$ is an efficient primer-template for reverse transcriptase from avian, murine, feline, and primate viruses.[207] None of the cellular DNA polymerases of prokaryotic or eukaryotic origin were able to use it. This may be the most specific primer-template for viral reverse transcriptase.

The divalent cation required for an optimal DNA synthesis varies with the primer-template used and also depends on the type of the virus (see Table 4). With $(dT)_{oligo}\cdot(rA)_n$, reverse transcriptases from avian type-C viruses[199] type-B virus, and MPMV[208] prefer Mg^{++} as divalent cation. Enzymes from most mammalian type-C viruses[199,201] prefer Mn^{++} over Mg^{++}. For $(dG)_{oligo}\cdot(rC)_n$ enzymes from avian type-C virus,[199] type-B virus[209] and MP-MV[196,209] prefer Mg^{++} over Mn^{++}, while the enzymes from mammalian type-C virus can use Mg^{++} and Mn^{++} with about equal efficiency.[199,201] For $(dT)_m\cdot(rA)_n$, SR-RSV enzyme[123] uses Mg^{++} and Mn^{++} with similar efficiency, AMV and MP-MV enzymes use only Mg^{++}[128,199] and those of mammalian type-C viruses[196,201] slightly prefer Mn^{++} over Mg^{++}. For $(rA)_n\cdot(rU)_m$ and $(dG)_{oligo}\cdot(Cm)_n$.[207] Mn^{++} is a better divalent cation for both avian and mammalian type-C virus enzymes.[49,199,207] For DNA primer-templates such as $(dA\text{-}dT)_n$ or activated DNA, Mg^{++} is generally a better divalent cation for all type-C virus enzymes tested.[123,199] For the same primer-template the optimal concentration of

divalent cation varies with the type of virus. In general, the optimal concentration for Mg^{++} ranges from 5 to 10 m*M*, while for Mn^{++}, it is from 0.1 to 1 m*M*. Another factor determining the preference and optimal concentration of the divalent cations is the configuration of the enzyme. For example, when R-MuLV is processed through a DEAE column, two forms of the enzyme, probably monomer and dimer, are eluted. The optimal concentration of Mn^{++} for the two forms are different when $(dT)_{oligo}\cdot(rA)_n$ is used (0.5 m*M* for the monomer and 1 m*M* for the dimer). Upon further purification, both forms of the enzyme become monomer and both have the same optimal concentration of Mn^{++}, i.e., 0.5 m*M*.[201]

As discussed previously, the optimal temperature is dependent on the thermal stability of the primer-template and also the species of virus. The pH optimum for the purified enzyme reaction is similar to that of the endogenous reaction. As for monovalent cations, Faras et al. reported that the purified enzyme from SR-RSV is strongly inhibited by relatively low concentrations of KCl, NaCl, or NH_4Cl,[123] but in many instances, an initial stimulation of activity is found followed by inhibition at higher concentrations.[124,196] In most cases, concentrations higher than 150 m*M* are inhibitory. A determination of the optimal concentration for each case is advisable.

The amount of DNA synthesis increases linearly with the concentration of primer-template but then decreases at very high concentrations. This inhibition of DNA synthesis by higher concentrations of primer-template can be partially overcome by the presence of nonionic detergent.[124] Possibly, the nonionic detergent prevents the primer-template from collape from the tertiary structure.

In the endogenous reaction, DNA-directed DNA synthesis is observed in addition to RNA-directed DNA synthesis.[58,141,210] These two activities can be separated by a high concentration of actinomycin D which suppresses DNA-directed DNA synthesis.[176,211] The purified enzyme is also able to catalyze both reactions.[123,128,145,199] Therefore, reverse transcriptase has apparently both catalytic functions or at least appears to have the capability of catalyzing both reactions.

b. DNA Synthesis Using Natural RNA

When the primer-template added to purified reverse transcriptase is viral HMW RNA, it is termed a "reconstruction reaction." The purified reverse transcriptase from avian viruses (such as AMV and RSV) is able to transcribe heteropolymeric portions of homologous and heterologous viral HMW RNA in a reconstruction reaction.[124,128,129,212] The fraction of the HMW RNA genome copied differs with various systems but is low in all, for example, Faras et al.[123] found it to be 3%. Avian viral enzymes use murine viral RNA as efficiently as avian viral RNA. The reverse transcriptase from mammalian viruses can also transcribe avian and mammalian viral HMW RNA with similar efficiency.[124,129-132] In all cases, all four deoxyribonucleotides are required for synthesis. In a reconstruction system, DNA synthesis is greatly enhanced by the addition of an exogenous oligomer such as $(dT)_{oligo}$ or $(dG)_{oligo}$.[124,149] The DNA products have been shown to link to the 3′-OH end of oligomer[129,150] and probably consist mainly of transcripts of the $(rA)_n$ or $(rC)_n$ (unpublished observation) regions of the viral RNA. Heat denaturation of HMW RNA greatly reduced the primer-template activity,[136,212] however, addition of oligomer to denatured RNA can partially restore the primer-template.[149,150] Apparently, these added oligomers serve as new initiators for DNA synthesis.

Several points are worth emphasizing in discussing transcription of HMW RNA by viral DNA polymerases: (1) The concentration of the enzyme should be sufficient. This is particularly important when only a small quantity of virus is available and the amount of enzyme is estimated only by its ability to use synthetic primer-templates; (2) The quality of HMW RNA should be carefully monitored. Probably one of the most critical tests is its ability to dissociate to 35S RNA upon heat treatment; (3) Optimal conditions for HMW RNA transcription are also important. In general, the divalent cation, Mg^{++} is preferable for enzymes isolated both from avian and mammalian type-C viruses,[123,128,213] but the MP-MV polymerase prefers Mn^{++}.[129] The optimum concentration of Mg^{++} is around 5 to 10 m*M*. Sometimes, addition of 1 m*M* Mn^{++} to a reaction mixture containing 5 m*M* Mg^{++} further enhances DNA synthesis;[212] (4) The products should be DNA and should be carefully analyzed to ensure that heteropolymeric portions of RNA and not the $(rA)_n$ portion are transcribed.[136,168]

Similar to the endogenous reaction, the pro-

ducts of the reconstructed reaction are also small (about 4 to 7S).[136,212] The products are bound to HMW RNA by hydrogen bonds and covalently linked to the 3'-OH end of the primer molecules.[136,212] The RNA-DNA linkage is rA·dA.[134,150] The primer is 4S RNA[137,139] which has tryptophan accepting ability[138] and slight methionine accepting ability.[138,195] This tRNA can be isolated from purified HMW RNA after heat treatment of the HMW (80°C).

Avian viral reverse transcriptase can also use many other natural RNAs as primer-template albeit not as efficiently as the 70S oncornavirus RNA, e.g., f2 viral RNA, Qβ viral RNA, bulk *E. coli* tRNA,[123,125] poliovirus RNA,[214] influenza virus RNA, tobacco mosaic virus RNA, and ribosomal RNA.[149] Some natural RNAs are poor templates for reverse transcriptase, but become very good primer-templates upon addition of oligomer. Examples include poliovirus RNA,[150] rabbit globin mRNA,[216,217] human globin mRNA,[267] and calf lens crytallin mRNA.[219] Globin mRNA transcripts approach 9 to 10S in size (the size of globin mRNA), and they are homologous to the mRNA. DNA synthesized in vitro from mRNA has been a useful probe in studying the regulation of gene expression, particularly of globin mRNA.

3. Structural Properties

Purified reverse transcriptase can be classified into three types based on molecular weight and subunit structure which we have arbitrarily defined as types I, II, and III. Type I reverse transcriptase is a single polypeptide with a molecular weight of about 70,000. Viruses containing this type of reverse transcriptase are mammalian type-C viruses (see Table 3 and References 51, 218, 220, 221) and avian REV.[222,223] Type II reverse transcriptase has two subunits with a molecular weight of about 170,000; the α subunit has a molecular weight of 70,000 and the β subunit has a molecular weight of about 110,000. All the avian leukosis-sarcoma viruses contain this type of reverse transcriptase. Type III reverse transcriptase has 1 subunit with a molecular weight of 110,000. The viruses containing this type of reverse transcriptase are the murine type-B virus, MP-MV, and the viper type-C virus (Table 3).

Three methods are frequently used:

1. **Velocity sedimentation in either a glycerol or sucrose density gradient** – With this method the gradient sedimentation should be performed in a high salt buffer to prevent the enzyme molecules from aggregating; in high salt buffer, the enzymes are relatively stable and sediment at about 4.5S for the type I enzyme, 6.5S for type II, and 5.5S for type III. In low salt buffer, the enzymes tend to aggregate and are somewhat unstable as judged by a poor recovery following gradient analysis.[196,222,224]

2. **Gel filtration** – As with the velocity sedimentation method, the molecular forms of reverse transcriptase are affected by the salt concentration of the buffer. Low salt buffer tends to form aggregates of the enzymes. For example, Nakajima et al. reported that aggregates of the enzymes with a molecular weight of 480,000 were obtained when highly purified R-MuLV enzymes were processed through sepharose 6B at low salt concentrations (0.05 *M* KCl) (without removing nucleic acid).[225] The same enzyme was found to have two lower molecular weight forms (135,000 and 70,000 daltons, respectively) when processed at high salt concentrations (0.5 *M* KCl). Another method of obtaining a monomer form is to perform agarose gel filtration in *6M* guanidine-hydrochloride.[226] By this method, the molecular weight of both R-MuLV and R-FeLV was found to be about 70,000. As well as the possibility of finding aggregate forms, it is still possible that polymerase may associate with nonpolymerase proteins. This could, of course, affect the molecular weight estimates. Another problem occurring both with the velocity sedimentation and gel filtration methods is the difficulty in obtaining accurate sedimentation values for the marker molecules. In fact, the molecular weight description of the monomer of type I reverse transcriptase varies in the literature from 50,000[202] to 90,000.[145]

3. **Polyacrylamide disc gel electrophoresis in the presence of 0.1% SDS** – This is the most accurate method for estimating size of single chains of polypeptides. By this method, it is generally agreed that the type I reverse transcriptase has a molecular weight ranging from 70,000 to 84,000[131,132], the α subunit of type II enzyme 69,000 daltons, the β subunit of type II enzyme 110,000 daltons, and the type III enzyme 110,000 daltons.

The configuration of the enzyme molecules sometimes depends on the method of purification. For example, when disrupted R-MuLV was processed through a DEAE cellulose column with a

shallow salt gradient, two peak activities were obtained. Peak I eluted at 0.05 *M* KCl and peak II at 0.14 *M*. Peak I has a molecular weight of 70,000 both in high and low salt gradients. Peak II enzyme consists of dimer or trimer forms at low salt and a monomer form at high salt in the presence of nonionic detergent. Peak II enzyme differs from peak I in some of its characteristics.[201] When both peak I and peak II enzymes were further processed through phosphocellulose columns, both eluted at a salt concentration of 0.2 *M* KCl.[201] Another example of a configurational change reported in the literature with respect to purification procedure is that of AMV. When the AMV polymerase was processed through a DEAE-cellulose column, a single peak activity was obtained. However, when this peak activity was further processed through phosphocellulose column, two peaks of activity were obtained.[227] Peak I contains only α subunit, while Peak II contains both α and β subunits. Both peaks contain reverse transcriptase and RNase H activities. Apparently, α subunit is the simplest functional unit for reverse transcriptase. The putative β subunit has yet to be isolated, and its function is not known. However, it was observed that the β "subunit" can change the mode of action of RNase H activity from random exoribonuclease activity to processive exoribonuclease activity.[228] Another factor that may affect molecular weight is digestion by a protease. Recently, Mölling[229] demonstrated that the β subunit can be converted in vitro to an α subunit plus a fragment with a molecular weight of 40,000 daltons by trypsin treatment or by aging of the enzyme at -20°C. The β to α ratio decreases from 4.1 to 0.43 upon trypsin treatment.[229] In fact, the fingerprint patterns of trypsin-partially-digested α and $\alpha\beta$ subunits were found to be nearly identical.[230,231] Similar observations of change of enzyme molecular weight (from 84,000 to 70,000) following protease treatment have also been made with Friend virus reverse transcriptase, a type I reverse transcriptase (Mölling, personal communication).

4. Immunological Properties

Antiserum against reverse transcriptase can be obtained either from animals bearing tumors induced by murine type-C viruses, or more frequently from animals immunized with purified reverse transcriptase.[232,233] Due to its specific neutralizing activity against reverse transcriptases, purified IgG has been a valuable reagent in distinguishing various types of reverse transcriptase and reverse transcriptase from cellular DNA polymerase, particularly the reverse transcriptase-like enzymes isolated from some human cells.[8,9,82,234] Table 5 summarizes the studies on the serological relationships among various sources of viral reverse transcriptase. Antibodies to about 10 different viral reverse transcriptase have been obtained in various laboratories. They are classified into six groups according to their antigenic relationship with their corresponding homologous enzymes:

1. Antibodies to GaLV, SSV, MuLV, and MuSV reverse transcriptase.[232,233,235]
2. Antibodies to FeLV and FeSV reverse transcriptase.[236]
3. Antibodies to cat (RD114)[213,236] and baboon (M7)[220] endogenous viral reverse transcriptase.
4. Antibodies to ALV reverse transcriptase.[232,237,238]
5. Antibodies to REV reverse transcriptase.[222]
6. Antibodies to MP-MV reverse transcriptase.[8,240]

Antibodies against reverse transcriptases of mammary tumor virus, syncytium-forming virus, and slow virus of sheep have not been reported. It is likely that some of these will become a new group. There are some variations in the reports regarding the antigenic relationships among different reverse transcriptases. For example, antibodies to SSV reverse transcriptase sometimes partially inhibit R-MuLV reverse transcriptase[8] but sometimes do not.[9] Similarly, antibodies to MuLV reverse transcriptase neutralize the FeLV reverse transcriptase with varying degrees of potency, and antibodies of RD114 reverse transcriptase sometimes inhibit SSV-1 and R-MuLV reverse transcriptase activity, but again sometimes do not. Normally, this variation does not occur in the same preparation of antiserum. Two factors contribute to this variation: (1) The host animals have different sensitivity in recognizing the antigenic determinants of inoculated antigens.[241,242] Therefore, it is important to try to prepare antisera in several species before the final determination of their antigenic relationship. (2) Purity of enzymes – unpurified

TABLE 5

Summary of Reports on Immunological Relatedness of Different Reverse Transcriptase

Source of reverse transcriptase	Source of antibody: GaLV	SiSV	MuLV MuSV	FeLV FeSV	RD114	M-7	AMV	RSV(RAV-O)	TDSNV	M-PMV
GaLV	++++	++++	++	0	+++		0			0
GBr 1-3	++++	++++	0			0	0			
SSV	++++	++++	++	0	0-+++	0	0			
C-MuV		++++	0-+							
MuLV	0-+	0-++	++++	++	0-+++	0	0			0
RD114	+	+	0-+	0	++++		0			0
M-7	0	0-+	0		+++	++++				
FeLV	0-+	0	0-+	++++	0-+		0			0
RaLV			+++	++			0			
HaLV			++	++			0			
PK (15) virus		++	+		+		0			
AMV	0	0	0		0		++++	+++	+	0
RSV(RAV-0)			0				++++	++++		
SR-RSV or B-RSV			0				++++	++++		
RAV-1 or RAV-2							+++			
B77							++++			
CSV							0	0	++++	
TDSNV							++	++	++++	
REV-T							++	++	++++	
MPMV	0	0	0	0			0			++++
MMTV	0		0	0			0			0
Visna virus			0				0			
Viper virus			0		0		0			
Foamy virus	0	0	0			0	0			
HL-23 virus	++++	++++	+	0-+	0-+	+	0			
Hep 12 virus		++++								
Class of antibody	I		II		III		IV		V	VI

*Degree of neutralization activity, ++++, ++++, strong; ++ = moderate; 0-+ = weak; 0 = no activity.

components may induce antibodies that non-specifically inhibit or stimulate reverse transcriptase activity. To obtain significant results regarding antigenic interrelationships among various reverse transcriptases, a well-defined assay system should be carefully monitored. The optimal conditions for antigen-antibody interaction, particularly the pre-incubation time,[157] the type and amount of non-specific protein carrier,[237] and the salt concentration should be determined.[222] Another method to study the interrelationships of the different reverse transcriptases is by a blocking test which is basically a measurement of competition between a given test enzyme and the homologous enzyme.[222,240] If a monospecific antibody is available, the radioimmune assay is another useful method.

At present several general conclusions can be reached regarding the interrelationships among antibodies from various sources of RNA tumor viruses and cellular DNA polymerases.[243]

1. Reverse transcriptases are only minimally or not detectably related to each of the cellular DNA polymerases.

2. Reverse transcriptases from type-C RNA tumor viruses are not detectably related to reverse transcriptases from type-B RNA tumor viruses.

3. Reverse transcriptases from type-C mammalian viruses are not detectably related to reverse transcriptases from avian type-C viruses.

4. Reverse transcriptases from type-C viruses obtained from various species of lower mammals (e.g., cats, rats, mice) are related but distinguishable.

5. Reverse transcriptases from the two distinct "families" of primate type-C viruses, i.e., the endogenous type-C virus of baboons vs. the horizontally "moving" type C virus from gibbon

ape leukemias and from woolly monkey sarcoma are distinct.

6. Reverse transcriptases from the gibbon ape leukemia virus (GaLV) and from the woolly monkey (simian) sarcoma virus (SSV) are very closely related.

7. Reverse transcriptases from the endogenous feline virus, RD114, is closely related to the endogenous primate virus (from baboons).

8. Reverse transcriptases purified from the fresh blood cells of some patients with acute myelogenous leukemia (AML) are very closely related to reverse transcriptase of SSV and GaLV.

9. The reverse transcriptases from three virus isolates obtained from blood and bone marrow of one patient with AML[82,416] are all closely related to reverse transcriptases of SSV and GaLV while a second viral component is related to BaEV components.

10. Reverse transcriptases purified from some patients with leukemia so far have not been found to be related to reverse transcriptase from any animal virus tested (Gallagher, R. and Gallo, R., unpublished results).

D. Enzymatic Activities Associated with Extracellular Particles

1. Virus Specific or Adventitious

Other than reverse transcriptase, some other enzymatic activities have been reported to be associated with virus particle preparations. These include DNA ligase activity,[244] DNA exonuclease activity,[244] nucleoside diphosphokinase activity,[245] nucleoside triphosphate phosphotransferase,[246] ATPase,[246-248] and protein kinase and phosphate acceptor proteins,[249,250] DNA endonuclease,[141,145] RNase,[122,125,251] RNA methylase,[252,253] nucleotide kinase,[247] ribonucleoside triphosphatase,[247] phosphoprotein phosphatase,[254] lactic dehydrogenase,[247] hexokinase,[247] AMV stimulatory protein,[255] aminoacyl tRNA synthetase,[248] ribonucleotide terminal transferase activity,[165,166] and ribonuclease H.[214,256,257] The location of these enzymes *in the virion* is defined based on the sensitivity of the activity to protease and the necessity of nonionic detergent treatment of the virions for detection of the activity. If the activity is detectable without nonionic detergent treatment and is sensitive to pronase, it is probably located on the outer envelope. If detection of the activity requires nonionic detergent treatment but is not detectable in the core fraction, it is probably located inside the outer envelope. If the activity associated with the particulate fraction is not affected by protease, requires detergent to detect, and bands at 1.25 g/ml in a sucrose density gradient, it very likely is located in the core. There are two reasons to suspect that many or all of the enzyme activities associated with the outer envelope are derived from cells: (1) All of the reverse transcriptase containing viruses bud from the host cell membrane and these membrane components can easily be associated with the budding virus; and (2) No "purified" virus is really free from cell debris. It is generally accepted that the enzymatic activities associated with the core have some significant function and those located inside the outer envelope may also have some viral specific function. However, it is important to emphasize that as long as our knowledge regarding the mechanism of viral replication and its genetic regulation is incomplete, it is presumptuous to assess a functional role to any enzyme activity which is not novel to the virus. In this section, the discussion will be limited to one enzyme, RNase H, which may very well be relevant to the reverse transcription pathway and at least in some cases may provide additional catalytic activity for the reverse transcriptase protein.

2. Ribonuclease H

There are at least two mechanisms to explain the conversion of RNA·DNA hybrids to double-stranded DNA during the course of synthesis of the proviral DNA. One is by peeling off the single-stranded DNA from the RNA·DNA hybrid, and the other is by an enzyme activity which is capable of degrading the RNA strand of a RNA·DNA hybrid. This type of specific nuclease has been found in calf thymus cells and is termed ribonuclease H.[258,259] Mölling and co-workers found such activity associated with AMV virions.[214] This observation was soon confirmed by Baltimore and Smoler[224] in AMV and was extended to some other mammalian type-C viruses by Grandgenett et al.[256]

a. Ribonuclease H from Avian Type-C Viruses

In avian myeloblastosis virus, RNase H co-purifies with reverse transcriptase through two ionic exchange chromatographies and glycerol gradient centrifugations.[224,227,260] The reverse transcriptase activity is eluted in two peaks from the phosphocellulose column containing α and $\alpha\beta$

subunits, respectively. Both peaks contain RNase H activity.[227] The RNase H activity is inhibited by antibody to AMV reverse transcriptase.[261,262] These findings strongly suggest that RNase H and reverse transcriptase activities reside on the same molecule. This is further supported by the fact that both activities were found in the core of the virions.[214] A firmer conclusion of this relationship was obtained by demonstrating that RNase H activity is present in α and $\alpha\beta$ subunits isolated in a nondissociating disc gel electrophoresis,[227] and that RNase H activity isolated from two temperature-sensitive mutants of reverse transcriptase of RSV, LA335, LA337 is also temperature-sensitive.[263,264] However, the functional sites of RNase H activity and reverse transcriptase activity appear to be separable for the following reasons: (1) RNase H activity both in endogenous reactions and with purified enzymes is inhibited more than 80% by 30 m*M* NaF and 150 m*M* KCl, while there is little effect on reverse transcriptase activity;[265] (2) Conversely, reverse transcriptase activity is stimulated by nonionic detergents but these have no effect on RNase H activity (our unpublished data); (3) Reverse transcriptase is much more heat labile than RNase H.[263,265]

The avian viral RNase H activity is exonucleolytic and the digested products are oligomers, predominantly dimers.[260] This is in contrast to the cellular enzymes from *E. coli*,[266] calf thymus cells,[260,267] chicken embryo cells,[260,261] human KB cells,[260] and human leukocytes,[268] which are endonucleolytic and from which the products are predominantly tetramers. These two findings have, therefore, been used as two criteria for distinguishing cellular and viral RNase H. However, in our judgment these criteria are not definitive because of the complexity of the enzyme kinetics *in vitro*. A more definitive answer may be obtained with antibodies to viral RNase H.

The role of the β subunit of viral reverse transcriptase is not known; it might have some regulatory role in the mechanism of action of RNase H activity. For example, Grandgenett et al.[228] reported that α subunit has a random exoribonuclease activity which results in the release of the substrate molecule after each chain scission while the $\alpha\beta$ molecule has processive exoribonuclease activity which completely degrades one polyribonucleotide chain prior to initiating hydrolysis of a second chain.

b. Ribonuclease H from Mammalian Type-C Viruses

Reverse transcriptase from mammalian viruses also contains RNase H activity, but the relative amount of RNase H activity to reverse transcriptase activity is less than that in avian viruses.[48,131,256,257,269] The amount of RNase H also varies with the type and preparation of the virus. For example, the RNase H activity reported for R-MuLV is only one fourth to one eighth that of AMV.[257] RNase H activity of F-MuLV is three to five times less than that of avian viruses (PR RSV-B, PR RSV-C), but two to three times more than that of Ki-MuSV (MuLV).[48,270] RNase H activity in M-MuLV, however, has been reported to be equivalent to that of AMV.[132] Since, in general, the activity is relatively low, it is not possible to detect activity if substrates of low specific activity are used for the assay.[221,257,270] Similar to avian virus enzyme, RNase H activity isolated from F-MuLV[131] and M-MuLV[132] was found to be random exoribonuclease. However, Mölling found that RNase H activity obtained from freshly purified F-MuLV enzyme (with a molecular weight of 84,000) contained processive exoribonucleolytic activity and this activity became random exoribonucleolytic when the molecular weight of the enzyme became 70,000 upon storage or partial trypsin digestion (personal communication). In contrast to the avian viruses, we found that RNase H activity is not present in the core of murine type-C viruses when the core structures are prepared with nonionic detergents and isolated in a sucrose density gradient.[257] We think that the lack of RNase H activity in the core probably is not due to a nonspecific inactivation or inhibition of RNase H activity during the experimental procedure since RNase H activity was detected in the core of AMV prepared by the same procedure. Furthermore, enzyme purified from the core of R-MuLV did not contain any detectable RNase H activity. We believe, therefore, that RNase H activity and reverse transcriptase do not reside on the same polypeptide molecule and not even in the same compartment of the virus. Two observations further support the conclusion that the RNase H is separable from reverse transcriptase in murine viruses: (1) The ratio of RNase H to reverse transcriptase tends to decrease during the purification procedures and varies with the batch of virus and the procedure of purification.[201] Olafsson et

al. were able to dissociate further RNase H activity from reverse transcriptase activity in a sucrose density gradient in the presence of high salt.[269] It is worth noting that some RNase H is easier to separate from reverse transcriptase from murine type-C virus than it is from avian viruses, and at least two RNase H activities were found in R-MuLV and K-MuSV(Ki-MuLV),[257] and M-MuSV(MuLV).[130] In addition to these observations, the larger form of R-MuLV reverse transcriptase contains much less RNase H activity than the LMW enzyme;[201] (2) Antibodies to mammalian viral reverse transcriptases do not inhibit RNase H associated with purified reverse transcriptase, although the purified enzyme used as antigen contained both activities (Wu and Gallo, unpublished data). This indicates that reverse transcriptase and RNase H have different antigenic determinants.

c. Miscellaneous Properties of RNase H

Virus particles contain many types of nucleases, and it is necessary to distinguish "pseudo" RNase H from true RNase H. Normally, RNase H is measured by the release of acid nonprecipitable radioactive oligoribonucleotides from RNA·DNA hybrids in which the RNA moiety is radioactively labeled. Three types of nuclease reactions imitate RNase H activity. One is a combination of a DNase and RNase, a second is an exonuclease III-like activity which degrades both RNA and DNA moieties of hybrid structures, and a third is a phosphodiesterase. In order to rule out these pseudo-RNase H activities, it is important to assay the nuclease activities with several substrates, for example: $[^3H]$ $(rA)_n \cdot (dT)_m$, $[^3H]$ $(rA)_n$, $[^3H]$ $(U)_n$, $[^3H]$ $(dT)_m \cdot (rA)_n$, and ϕX174 DNA · $[^3H]$ RNA.[257,268] If only a true RNase H is measured, only $[^3H]$ $(rA)_n \cdot (dT)_m$ and ϕX174 DNA · $[^3H]$ RNA are expected to release soluble oligoribonucleotides. In some studies, results with $[^3H](dT)_m \cdot (rA)_n$ as substrate were not reported. Although $[^3H](rA)_n \cdot (dT)_n$ is commonly used as substrate, it is not the best for detecting low activity. ϕX174 DNA·$[^3H]$ RNA and calf thymus DNA·$[^3H]$ RNA are more efficiently utilized than the synthetic substrates.[257,262] RNase H activity is sensitive to salt even as low as 0.1 *M*, and therefore its presence can be missed if care is not taken to adjust salt concentrations when assays are made from samples eluting from columns. Another factor affecting the assay of this enzyme is divalent cation. Both Mn^{++} and Mg^{++} are suitable for enzyme catalytic action, but they have different optimal concentrations. The range reported for Mn^{++} is 0.3 to 0.8 m*M*. Mg^{++} optimum is about 5 m*M*.[48] The molecular weight of mammalian viral RNase H is not known, although there are indications that it is smaller than but very close to the size of reverse transcriptase.[269] Cellular RNase H is, in fact, about 70,000 to 80,000 daltons.[260,268] The discovery of RNase H has prompted some speculations regarding the mechanism of double-stranded proviral DNA synthesis. The models proposed by Mölling et al.,[214] Keller and Crouch,[261] and Leis et al.[276] all require participation of a host endonucleolytic RNase H as well as the viral nuclease. They do not explain the conversion of the double-stranded RNA primer region to double-stranded DNA.

E. Criteria for Viral Reverse Transcriptase

In view of the frequent use of inadequate criteria to define intracellular reverse transcriptase and sometimes extracellular particle enzymes, it is important to examine carefully the criteria which are useful and/or specific for the definition of reverse transcriptase. We use two approaches: one is biochemical, and the other is immunological. Table 6 lists those criteria employed in the authors' laboratory. No one criterion is absolute.

1. Biochemical Criteria

a. Density

The intact type-C virus particle has a density of about 1.16 g/ml,[118] type-B particles about 1.18 g/ml,[227] and cores about 1.25 g/ml.[118,119] These densities are not unique to virus particles. Some cellular membrane components may have similar densities. The density of virus particles also varies with many conditions such as the amount of membrane lipoprotein and cellular nucleic acids associated with the particles and the age of the particles. It can range from 1.13 to 1.19 g/ml. The association of cellular components can be reduced by repeated purification in equilibrium density gradients or by very mild treatment of the virus particles with nonionic detergent. Aging or freezing and thawing tend to disrupt some viral particles. The partially disrupted particles are generally heavier than that of intact particles, since the core fraction of the particle has a density of about 1.25 g/ml[118,119] and the ribonucleoprotein complex is more than 1.3 g/ml.[120,121] In

TABLE 6

Criteria for Reverse Transcriptase

Biochemical criteria

1. The enzyme activity is detected in a particle fraction with a density of 1.15–1.18 g/ml and shifted to above 1.25 g/ml upon treatment with adequate concentration of nonionic detergent.
2. The endogenous reaction requires all four deoxyribonucleoside triphosphates. It is at least partially sensitive to RNase and resistant to actinomycin D, and product analysis reveals that the DNA is hydrogen bonded to a large RNA and covalently attached to a small RNA.
3. The purified enzyme is able to use $(dG)_{oligo} \cdot (rC)_n$, $(dG)_{oligo} \cdot (rCm)_n$, and $(dT)_{oligo} \cdot (rA)_n$ as primer-template, but not $(dT)_{oligo} \cdot (dA)_n$ and $(rA)_{oligo} \cdot (dT)_n$. When unpurified enzyme is used, the $(dT)_{oligo} \cdot (rA)_n$ to $(dT)_{oligo} \cdot (dA)_n$ ratio is high when Mg^{++} or Mn^{++} is used as divalent cation.
4. The enzyme is capable of transcribing heteropolymeric portions of viral HMV RNA.
5. The enzyme activity is stimulated by nonionic detergent when $(dT)_{oligo} \cdot (rA)_n$, $(dT)_m \cdot (rA)_n$, activated DNA, or $(dG)_{oligo} \cdot (rC)_n$ is used as primer-template.
6. The size of the enzyme is about 70,000 daltons for mammalian type-C viruses, 160,000 daltons for most avian type-C, and 110,000 daltons for type-B virus or MPMV.

Immunological criteria

The enzyme activity may be inhibited by purified IgG made against some known viral reverse transcriptase and not significantly inhibited by IgG against cellular DNA polymerases.

these high density regions, the purity of viral components increases. Therefore, the presence of the enzyme activity in the core fraction prepared by nonionic detergents is a useful criterion for reverse transcriptase. The authors generally have found intracellular reverse transcriptase in the cytoplasm in the post-mitochondrial and mitochondrial particulate fraction.[6,9-11,108,197] The density has varied and probably depends on many factors, especially the method of cell disruption, but generally it has been between 1.13 and 1.19 g/ml.

b. RNA-directed Reaction

The first step to demonstrate that a given enzyme catalyzes RNA-directed DNA synthesis reaction is to show that all four deoxyribonucleoside triphosphates, namely dATP, dGTP, dCTP, and dTTP, are required for optimal DNA synthesis in an endogenous reaction. Omission of one or more triphosphate should then reduce the amount of DNA synthesized. One can rule out that the polymerization of deoxyribonucleoside monophosphate is not due to terminal transferase by using only one of the nucleoside triphosphates. Another conventionally used criterion is the RNase sensitivity. As pointed out previously, this is not an absolute criterion and this test should be performed in the presence of high salt and relatively low concentration of RNase. However, definite proof that the reaction is RNA-directed rests on the product analysis (see Section IIB2). Although a proper product analysis in itself does not necessarily mean that the activity is from viral reverse transcriptase, there is as yet no other known enzyme which catalyzes an endogenous RNA-directed DNA synthesis.

c. Synthetic Primer-template Specificity

Among many synthetic DNA·RNA hybrid primer-templates, $(dT)_{oligo} \cdot (rA)_n$ and $(dG)_{oligo} \cdot (rC)_n$ are probably the most efficient for reverse transcriptase. Since purified reverse transcriptase does not use $(dT)_{oligo} \cdot (dA)_n$ while some cellular DNA polymerases do, the authors of this article routinely compare the utilization of $(dT)_{oligo} \cdot (rA)_n$ with that of $(dT)_{oligo} \cdot (dA)_n$. Though cellular DNA polymerase γ[156] and β[157] use $(dT)_{oligo} \cdot (rA)_n$ as template, they can also use $(dT)_{oligo} \cdot (dA)_n$. Therefore, a high ratio between the utilization of $(dT)_{oligo} \cdot (rA)_n$ to the utilization of $(dT)_{oligo} \cdot (dA)_n$ is an additional criterion for the presence of viral reverse transcriptase. This ratio should be measured both with Mn^{++} and with Mg^{++} as divalent cation. If Mg^{++} is used as divalent cation, a slight contamination with cellular enzyme can easily reverse the ratio, since cellular polymerases use $(dT)_{oligo} \cdot (dA)_n$ much more efficiently than reverse transcriptase. The use of $(dT)_m \cdot (rA)_n$ as primer-template to detect reverse transcriptase activity[59,147] is not valid bacause some cellular enzymes (both prokaryote and eukaryote) use it very well.[99,206,281-283] Another useful criterion to distinguish cellular DNA polymerases, particularly DNA polymerase γ from reverse transcriptase, is that cellular DNA polymerases are able to synthesize $(rA)_n$ using $(rA)_{oligo} \cdot (dT)_n$ as primer-template[158] while

none of the viral reverse transcriptase tested is able to use this primer-template (M. Robert-Guroff, personal communication).

$(dG)_{oligo}\cdot(rC)_n$ was thought to be completely specific for viral reverse transcriptase. However, recently it was shown that purified cellular DNA polymerase γ can use this primer-template albeit with poor efficiency.[158,284] However, since all reverse transcriptases use this primer-template efficiently, the authors use it as an important criterion, although again not an absolute one. Recently, Gerard et al. reported that $(dG)_{oligo}\cdot(rCm)_n$ is specific for viral reverse transcriptase both from type-C[207] and type B particles.[285] Utilization of $(dG)_{oligo}\cdot(rCm)_n$ should, therefore, be a useful criterion for distinguishing viral transcriptase from cellular polymerase. We confirmed this observation.

d. Natural RNA Primer-template

The authors think the most specific primer-template is the HMW RNA from viruses. Detection of DNA·RNA hybrids in a short-term reaction with this RNA is a direct demonstration that a given enzyme is capable of using the HMW RNA as primer-template. This is usually done by analyzing the products in a cesium sulphate density equilibrium gradient or by measuring the resistance of product-primer-template complex to S1 nuclease. Since viral HMW RNA contain a tract of $(rA)_n$, it is necessary to demonstrate further that the products are not $(dT)_n$ and are complementary to heteropolymeric portions of the HMW RNA. Reduction of DNA synthesis following omission of one or more nucleotides and the demonstration of DNA synthesis by using labeled deoxyribonucleoside triphosphate other than dTTP are some indications of transcription of non-$(rA)_n$ regions. More definitive evidence can be obtained by hybridizing the DNA products back to HMW RNA in the presence of a relatively high concentration of $(rA)_n$. Although purified *E. coli* polymerase I transcribes HMW RNA,[13] the amount of *E. coli* DNA polymerase I required for this transcription is about 200-fold more than that of reverse transcriptase. To date, eukaryotic DNA polymerases (α, β, and γ) have not been shown to transcribe viral RNA. Therefore, transcription of viral HMW RNA may be probably the most specific criterion.

The use of globin mRNA in the presence of $(dT)_{oligo}$ is another useful criterion for reverse transcriptase since cellular DNA polymerases apparently may not transcribe heteropolymeric portions of mRNA even if high concentrations of these enzymes are used.[113]

e. Stimulation by Nonionic Detergent

Thompson et al. observed that reverse transcriptase activity is enhanced by nonionic detergent.[287] This is not due simply to protection from a nonspecific loss of enzyme, but rather is due to an enhancement of the availability of some primer-templates which may have a tendency to collapse in solution, for example, $(dT)_{oligo}\cdot(rA)_n$, $(dG)_{oligo}\cdot(rC)_n$ and activated DNA. This stimulatory activity by nonionic detergents may be unique to reverse transcriptase. The activity of cellular DNA polymerase was not enhanced by the presence of nonionic detergent. This could be an additional criterion to help distinguish reverse transcriptase from other cellular enzymes.

f. Size

As discussed in Section II.C.3 reverse transcriptase can be divided into three types based on their molecular size. Among them the molecular weight of the subunit structure is unique for each type of the enzyme. Since the enzyme tends to aggregate in a low salt condition, an estimate of the molecular weight should be performed in a high salt condition or in the presence of detergent. The finding of an appropriate molecular weight for an enzyme in question is a helpful criterion in diagnosing it as reverse transcriptase.

2. Immunological Criteria

As previously discussed, reverse transcriptase can be subdivided into six groups according to their immunological properties. Inhibition of polymerase activity by IgG to reverse transcriptase from a known RNA tumor virus indicates a relationship exists between the polymerases. Generally, antibody to viral reverse transcriptase does not neutralize cellular DNA polymerases[9,11,157] and vice versa[157,311]. Therefore, lack of inhibition by antibodies to cellular DNA polymerases is a useful indication of the presence of reverse transcriptase.

III. INTRACELLULAR REVERSE TRANSCRIPTASES

Following the initial finding of a reverse transcriptase-like activity in human leukemic cells by Gallo et al.,[5] many studies have been carried out to detect a "virus-specific" enzyme in a variety of normal and malignant tissues and in virus

infected and uninfected cells (Table 1). The purpose of these studies includes: (1) to obtain evidence for the presence of an RNA tumor virus in certain neoplastic cells of man; (2) to understand the mechanism of viral replication in viral and transformed cells (3) to elucidate the role, if any, of reverse transcriptase in development and differentiation; (4) to determine if detection of the activity is useful for diagnostic and prognostic modalities in human malignant diseases; and (5) to study the relationship between cellular DNA polymerases and viral reverse transcriptase. In many of these studies, strict criteria for reverse transcriptase were not applied and therefore there was confusion between authentic viral enzymes and some cellular enzymes. Reverse transcriptase from human leukemic cells is the only well-studied intracellular reverse transcriptase[6,9] described to date from cells not *known* to be producing virus. It is, of course, easier to isolate viral reverse transcriptase from virus-producing cells.[99,100,421]

Reverse transcriptase has been identified in two locations in cells, the cytoplasm and the cisternae. The enzyme in the cytoplasm is associated with the post-mitochondrial pellet fraction and in "A" particles is found in cisternae. The relationship, if any, between these two reverse transcriptases from these two particulate fractions is not known. For convenience, the reverse transcriptase associated with these two particulate fractions will be discussed separately.

It is obviously much more tedious to obtain purified reverse transcriptase from cells than it is from virus. This is due, among other things, to the presence of cellular DNA polymerases (at least one which has properties similar to those of reverse transcriptase), the distribution of reverse transcriptase in various subcellular compartments, the low amount of reverse transcriptase per unit of protein (in the case of nonproducer cells), and the presence of nucleases and proteases. In order to distinguish reverse transcriptase from cellular DNA polymerases, the major properties of the cellular enzymes must be known. They are summarized below.

A. Eukaryotic Cellular DNA Polymerases

Four distinct cellular polymerases have been isolated from a variety of mammalian cells. They are DNA polymerase α, DNA polymerase β, DNA polymerase γ, and mitochondrial DNA polymerases. Some relevant biochemical and immunological properties of these enzymes are listed in Table 7. To prevent confusion, the old nomenclature for these enzymes is listed in Table 8. For recent detailed reviews see Reference 289.

DNA polymerase α is found in the cytoplasmic fraction of many tissues including human leukocytes and cultured cells,[99,206,283,290] rat liver[295-297] rabbit and mouse cells,[289,299,300] Morris hepatoma,[301] rat ascites hepatoma cells,[302,303] calf thymus,[304,309] and chicken embryonic cells.[222,305] The amounts of this enzyme increase when cells are in a proliferative stage.[274,295,300] Presumably then, this enzyme is required for cell proliferation. DNA polymerase α has been called the high molecular weight DNA polymerase; it sediments at 6 to 8S. The reported molecular weight ranges from 90,000 to 250,000 daltons.[99,206,290,298,304] The high molecular weight form probably represents an aggregate form.

Recently, Yoshida et al. have found interconvertible molecular species of cytoplasmic DNA polymerase from calf thymus.[304] Since nucleic acids were not removed prior to fractionation by gel filtration, the results may be explained by association of enzyme with nucleic acids. The basic subunit of these aggregates has a molecular weight of 90,000,[304] which agrees with the recent findings of Sedwick et al.,[283] Smith et al.,[157] and Chang et al. (personal communication).

In general, the preferred divalent cation is Mg^{++} and the preferred primer-templates are DNAs such as activated DNA and $(dA\text{-}dT)_n$. The enzyme does not use $(dG)_{oligo}\cdot(rC)_n$, natural RNAs, or mitochondrial DNA as primer-template. In the presence of Mg^{++}, DNA polymerase α uses $(dT)_m\cdot(A)_n$ and $(dT)_{oligo}\cdot(dA)_n$. $(dT)_{oligo}\cdot(rA)_n$ is used with very poor efficiency if at all. This enzyme is relatively sensitive to N-ethylmaleimide ($Ki=10^{-5}$ M)[206,283,289,293] and to salt.[206,309] The pH optimum of this enzyme is 7.5 to 8.[296] Recently, Smith et al. prepared antibodies (in rats) to DNA polymerase α isolated from human lymphocytes.[157] The purified IgG does not inhibit the activity of DNA polymerase β from the same cells or different types of cells.[157] Chang and Bollum[310] originally found a partial cross reaction of polymerase α and β with antisera prepared in rabbits. However, in agreement with Smith et al. and Weissbach et al.,[311] Bollum with a different antiserum (personal communication), more recently did not find the cross reaction. The original antigen of Chang and Bollum must either have been contaminated with DNA polymerase β or more likely the anti-

TABLE 7

Some Biochemical Properties of Various Cellular DNA Polymerases

Properties	DNA Polymerase α	DNA Polymerase β	DNA Polymerase γ	Mitochondrial DNA polymerase	Human leukemia and mammalian viral reverse transcriptase
Cellular location	Cytoplasm	Nucleus and/or cytoplasm	Nucleus and/or cytoplasm	Mitochondria	Cytoplasm
Molecular weight* (Daltons × 10^{-5})	0.9 (6–8S)	0.4 (3.5S)	1.0 (6.1–6.3S)	1.0	0.7 (4.5S)
Utilization of Template-primer**					
Activated DNA	++++ (Mg^{++})	++++ (Mg^{++})	+++ (Mg^{++})	++++ (Mg^{++})	++ (Mg^{++})
$(dT)_{oligo} \cdot (dA)_n$	+ (Mg^{++})	++ (Mg^{++})	++ (Mg^{++})		
	+++ (Mn^{++})	++++ (Mn^{++})	± (Mn^{++})	–	± (Mn^{++})
$(dT)_{oligo} \cdot (rA)_n$	± (Mg^{++})	– (Mg^{++})	+ (Mg^{++})		
	+ (Mn^{++})	+++ (Mn^{++})	+++ (Mn^{++})	–	++++ (Mn^{++})
$(dG)_{oligo} \cdot (rC)_n$	–	–	± (Mn^{++})	–	+++ (Mn^{++})
$(rA)_{oligo} \cdot (dT)_n$	++ (Mn^{++})	+ (Mn^{++})	+++ (Mn^{++})	N.R.	–
70S RNA	–	–	± (Mn^{++})	N.R.***	++ (Mn^{++} or Mg^{++})
Mitochondria DNA	–	–	–	+++ (Mg^{++})	N.R.
Nonionic detergent stimulation	no	no	no	N.R.	yes
N-ethylmaleimide inhibition	Sensitive	Insensitive	Intermediate	Insensitive	Sensitive

*The value from the smallest subunit reported is used.
**++++: High efficiency; ++ – +++: moderate utilization; +: slight utilization; ±: marginal utilization; and –: not used.
***N.R.: Not reported.

TABLE 8
Summary of Synonym of Eukaryotic Cellular DNA Polymerase

α*	β*	γ*	Reference
I	II	III	Smith and Gallo[206] Lewis et al.[156]
D-II	D-I	R-I or R II	Weissbach et al.[293] Fridlender et al.[281] Spadari and Weissbach[158]
C or N2	N1		Sedwick et al.[283]
High molecular weight	Low molecular weight		Chang and Bollum[298]
(6–8S)	(3.3S)		Chang and Bollum[295]
(maxi)	(mini)		Coleman et al.[290]

*See Reference 110.

genic determinant to which the host animal responded was more broadly reactive. The IgG to DNA polymerase α also does not inhibit DNA polymerase γ[156] nor any of the virus-related reverse transcriptases.[8,9,157] This antigenic property can therefore be used to distinguish DNA polymerase α from reverse transcriptases and probably other cellular DNA polymerases.

DNA polymerase β has been found in the cytoplasm and in the nucleus of a variety of tissues. These reports include human leukocytes and cultured cells,[99,206,283,290,293,294] rat liver,[295-297] rabbit tissues,[282,298] calf thymus,[312,313] murine tissues,[299,300] rat ascites hepatoma tissues,[302,303] Morris hepatoma tissue,[301] and chicken embryonic cells.[222,305] DNA polymerase β has a molecular weight of about 40,000 to 50,000 daltons. It sediments at about 3.5S. Activated DNA and $(dA\text{-}dT)_n$ are the most efficient primer-templates for this enzyme in the presence of Mg^{++}, while $(dT)_{10}\cdot(dA)_n$ and $(dT)_m\cdot(rA)_n$ are the best primer-templates in the presence of Mn^{++}. $(dG)_{oligo}\cdot(rC)_n$, HMW RNA, and mitochondrial DNA are not utilized. Several factors affect the template specificity of the enzyme, for example, the purity of the enzyme, divalent cation, and salt concentration. The enzyme activity is stimulated by high ionic strength. The optimal salt concentration is between 0.1 *M* and 0.2 *M* NaCl. The pH optimum of the enzyme is 8.5 to 9.0.[289] The enzyme activity is not stimulated by nonionic detergent.[124] It is not inhibited by NEM[206,290] by antibodies to DNA polymerase α,[157,311] nor by antibodies to viral reverse transcriptase.[8,9] The only antibody to DNA polymerase β available is from chicken cells.[222] This antibody is able to neutralize the activities of avian DNA polymerase α and β from various strains of chickens and rat cells, DNA polymerases of REV-T and TD-SNV, and rat DNA polymerase β, but not rat DNA polymerase α. Many of the biochemical and immunological properties of DNA polymerase β make it easy to distinguish it from reverse transcriptase.

DNA polymerase γ is found in the nucleus and sometimes in the cytoplasm. Some of its properties are similar to those of reverse transcriptase, and therefore a thorough familiarity with the properties of this enzyme is advisable when examining cells for reverse transcriptase. This enzyme, in fact, was probably the activity detected earlier by a number of workers in various cells and confused with reverse transcriptase. It was first named R-DNA polymerase due to its ability to copy the RNA strand of synthetic RNA·DNA homopolymer hybrids.[281] Since both L cells and HeLa cells used in these studies might have been producing low titers of a RNA tumor virus, the evidence that this was a new polymerase was not final. However, the presence of this DNA polymerase as a distinct enzyme was verified when Lewis et al.[99,156] and McCaffrey et al.[291] isolated this enzyme from human normal and leukemic leukocytes and Evans et al.[299] from mouse spleen. These studies demonstrated that DNA polymerase γ is distinct from DNA polymerase α and β, and also from viral reverse transcriptase.[156] This was further confirmed when Spadari and Weissboch purified this enzyme.[158] DNA polymerase γ in our studies has a molecular weight of approximately 100,000. It is able to

copy activated DNA, $(dT)_{oligo} \cdot (dA)_n$, and $(dA\text{-}dT)_n$ with moderate efficiency. It also efficiently uses $(dT)_{oligo} \cdot (rA)_n$ and $(dT)_n \cdot (rA)_n$ in the presence of Mn^{++}. However, with Mg^{++}, it is virtually inactive with these primer-templates. The optimal salt concentration for DNA polymerase γ is about 100 mM. If the salt concentration is decreased the capacity to use $(dT)_{oligo} \cdot (rA)_n$ is diminished while ability to use $(dT)_{oligo} \cdot (dA)_n$ is enhanced. The authors believe an important criterion in distinguishing polymerase γ from reverse transcriptase is to determine the activity with $(dT)_{oligo} \cdot (dA)_n$ with Mg^{++} and Mn^{++}. Polymerase γ will use $(dT)_{oligo} \cdot (dA)_n$ in Mg^{++}, but under no circumstances does reverse transcriptase use $(dT)_{oligo} \cdot (dA)_n$ in the authors' experience. Recently Spadari and Weissbach reported that the polymerase γ from HeLa cytoplasm could be further fractionated into two peak activities in both phosphocellulose and hydroxylapatite columns.[158] One peak, $\gamma 1$, is able to transcribe $(dT)_{oligo} \cdot (rA)_n$, but not $(dG)_{oligo} \cdot (rC)_n$, $(dC)_{oligo} \cdot (I)_n$ and $(dA)_{oligo} \cdot (U)_n$. However, the other peak, $\gamma 2$, is able to transcribe all of these primer-templates with relatively high efficiency. The first peak, $\gamma 1$, is similar to the DNA polymerase γ found by Lewis et al.[156] The second peak, $\gamma 2$, is a new activity. Apparently, both enzymes are antigenically unrelated to reverse transcriptase isolated from SSV-1, MPMV, and R-MuLV.[158]

Mitchondrial DNA polymerase has been isolated and purified from mitochondria of HeLa cells[306,315] and rat liver.[307,316,317] The molecular weight of the enzyme is about 100,000 which is significantly larger than that of DNA polymerase β, but similar to those of DNA polymerases α and γ. This enzyme can also efficiently use activated DNA as template-primer. However, mitochondrial DNA polymerase is distinguished from the other DNA polymerases by the fact that it can use native mitochondrial circular DNA as template-primer (initiation of nick by an endonuclease). Mitchondrial DNA polymerase does not copy $(dT)_{oligo} \cdot (rA)_n$, $(dT)_{oligo} \cdot (dA)_n$, $(dA)_{oligo} \cdot (rU)_n$, $(dG)_{oligo} \cdot (rC)_n$ and $(dC)_{oligo} \cdot (rI)_n$.[306] This is in contrast to DNA polymerase γ which uses all of these template-primers to varying degrees. Similar to DNA polymerase β, the mitchondrial polymerase is relatively insensitive to NEM.[306] There are no studies regarding the utilization of natural RNA nor any on the antigenic relationships of this enzyme to other DNA polymerases. However, this enzyme is easily distinguished from reverse transcriptase by many criteria.

B. Intracytoplasmic Reverse Transcriptase

From virus-producing cells, it is a relatively simple task to isolate and purify virus-related reverse transcriptase. However, from the cells not producing virus, if present at all, its detection and isolation is very difficult. Intracytoplasmic reverse transcriptase is usually found in the high speed (90,000 xg) particulate fraction from the post-mitochondrial fraction. This particulate fraction has provided the most successful method to detect and isolate reverse transcriptase for the authors of this article. An extensive study of the nature of the cytoplasmic particulate fraction has been carried out with human leukemic blood cells.[10] This can serve as a model system for the study of intracellular virus-like particles and associated reverse transcriptase.

1. Reverse Transcriptase from Human Leukemic Cells

Initially, reverse transcriptase-like activity was observed in three cases of acute leukemia but not in PHA stimulated lymphocytes.[5] The activity was detected in nucleic acid-free "cytoplasmic" supernatant, but subsequently, the high speed cytoplasmic pellet fraction was identified as the best subcellular fraction.[6,318] DNA synthesis can be obtained in the cytoplasmic pellet with endogenous primer-template. The reaction is sensitive to RNase and the DNA product bands as an RNA·DNA hybrid. The enzyme purified from this fraction is able to transcribe viral RNA,[6,7] and in at least some cases of adult AML, is is antigenically related to reverse transcriptase from certain primate type-C viruses.[8-10] The biochemical studies were extended to all types of leukemia,[319] but in our studies not all leukemic cases were found to contain reverse transcriptase. To date, it is only with AML cells that the immunological relatedness to known viral reverse transcriptase has been found. The reproducibility of detection has been improved by banding the cytoplasmic pellet fraction in sucrose density gradients and isolating the particulate region from a density of around 1.16 to 1.18 g/ml.[9] The specific activity of the enzyme can be enhanced by repeated banding in a sucrose density

gradient.[10] With this modified procedure, in some cases it is possible to detect reverse transcriptase activity with about 109 nucleated cells. It is important to emphasize that with this relatively small amount of leukemic cells, one has to isolate the cytoplasmic pellet in order to detect reverse transcriptase activity, and it has not been possible to detect this enzyme activity by isolating it from "nucleic acid free" cytosol[10] (B. Lewis and R. C. Gallo, unpublished observation). In our experience, reverse transcriptase is detectable in only 20% to 30% of patients with leukemia. Moreover, we emphasize again that it is only with adult AML that the immunological relationship to known primate type-C viruses has been demonstrated so far, and even in adult AML, only about one-third of all patients examined have detectable enzyme. This is in contrast to the data of Spiegelman and colleagues who have reported detection of reverse transcriptase in virtually all cases of leukemia examined.[13]

a. Purification

The procedures described here are those that isolated the cytoplasmic particulate fraction before solubilization and purification of reverse transcriptase.

(1) Cytoplasmic Particles

To prepare the cytoplasmic particulate fraction, fresh human leukemic cells are mechanically disrupted. The nucleic acid and mitochondria are removed by a differential centrifugation at 1,000 and 12,000 xg, respectively. The post-mitochondrial supernatant is further centrifuged at a high speed (90,000 xg) to obtain the post-mitochondrial pellet fraction. The pellet fraction is then banded from one to three times in an equilibrium sucrose density gradient and the RNase sensitive endogenous DNA synthesizing activity is detected from fractions with a density of approximately 1.15 to 1.19 g/ml. These fractions contain reverse transcriptase and nucleic acids. To enhance the specific activity of the endogenous reaction, the pellet fractions are banded repeatedly in the sucrose density gradients.[10] These particles have some properties similar to those of type-C RNA tumor viruses (see Reference 320). For example, in some cases these particles can be converted to an entity with a density of approximately 1.25 g/ml by treatment with a nonionic detergent. This density is similar to that of the virus cores. The size of the 1.16 g/ml particles varies from 500S and 1,000S and sometimes they form larger aggregates as judged by gel filtration.[10] Virus-related nucleic acid (RNA) and virus-related reverse transcriptase are complexed in these particles. Like RNA tumor viruses they give rise to an endogenous DNA synthesis in the test tube. Most of this reaction is resistant to actinomycin D, but it is sensitive to RNase to some degree. The degree of resistance to actinomycin D and sensitivity to RNase varies from case to case and from preparation to preparation. In general, a better result is obtained when the particles are purified more extensively by repeated banding.[10] The products from the endogenous reaction are covalently linked to an RNA primer and associated with HMW RNA through hydrogen bonds.[6] Similar to the products of a viral endogenous reaction, the DNA products are small but contain sequences complementary to RNA of some known type-C primate oncogenic viruses (SSV-1 and GaLV[12] and R-MuLV[13]). Additional evidence that the cytoplasmic particles contain virus-related RNA is based on positive molecular hybridization between particle RNA and DNA products from known type-C primate oncogenic viruses. The size of the virus-related RNA in the particle is not known with certainty, but the presence of HMW RNA is suggested by a positive "simultaneous detection" assay,[13,127] and more recently by direct labeling of the RNA.[278] There is evidence indicating that reverse transcriptase isolated from these particles is viral-related. This will be discussed below.

(2) Solubilization of Reverse Transcriptase

The advantages of isolating intracellular reverse transcriptase from cytoplasmic particles are

1. It requires as little as 10^9 cells to obtain a reasonable amount of enzyme activity.
2. It is easier to obtain enzyme with relatively good specific activity.
3. One can obtain the presumed native RNA template and prepare labeled DNA probes.

The enzyme can then be further purified by conventional purification procedures such as DEAE cellulose and phosphocellulose chromatography and Sephadex G-200 gel filtration.[6] About a 200-fold purification was obtained over

that of the pellet fraction. Alternatively, the enzyme can be purified by a Sepharose 4B gel filtration step.[9,11] This procedure has been especially effective with small amounts of cells. The solubilized and partially purified reverse transcriptases exhibit the biochemical and immunological properties of known murine and primate viral reverse transcriptases (see Table 6 for the criteria of viral reverse transcriptase and recent summary by Gallo[109]). The enzyme significantly prefers $(dT)_{oligo} \cdot (dA)_n$ over $(T)_{oligo} \cdot (dA)_n$ as primer-template and can efficiently copy $(dG)_{oligo} \cdot (rC)_n$.[6,9] These activities are stimulated by nonionic detergent.[124] Similar to many DNA polymerases, the human virus-like DNA polymerase can copy activated DNA, $(dT)_m \cdot (rA)_n$ and $(dA\text{-}dT)_n$.[6] Another important feature of this enzyme is its ability to transcribe the heteropolymeric portions of both avian and mammalian viral HMW RNA.[6,7] The molecular weight of human reverse transcriptase was found to be 130,000 when the enzyme was solubilized in low salt buffer.[6] However, a low molecular weight form with a molecular weight of 70,000 was obtained when the enzyme was solubilized in the presence of high salt buffer and Triton X-100.[9,11] The two forms of enzyme are interconvertible. The HMW forms can be converted to LMW form by treatment with high salt and nonionic detergent (presumably to remove lipoid components) and LMW form can be reaggregated to the HMW form by a dialysis against a low salt buffer.[11] Primer molecules are required for this reaggregation. These two forms of the enzyme exhibit some variant biochemical and immunological properties. These are (1) the HMW form utilizes synthetic primer-templates less efficiently than the LMW form: (2) the HMW form transcribes HMW RNA in the absence of synthetic primer relatively more efficiently than the LMW form of reverse transcriptases.[11,201] (3) the HMW form is not inhibited by the antibody to primate oncogenic viral DNA polymerase. This variation in the enzyme following conversion of molecular weight may have some functional significance *in vivo*. Apparently, this phenomenon is not unique to human intracellular reverse transcriptase, since it has also been observed in the intracellular transcriptase isolated from lymphoblasts deliberately infected by and producing GaLV,[11] murine cells producing R-MuLV,[201] and the extracellular viral reverse transcriptase from R-MuLV (Reference 201 and unpublished observation).

b. Evidence of Viral Gene Expression or Virus Infection

(1) General Criteria

The presence of viral components in tumor cells might be indicative of a previous history of virus infection and/or expression of an endogenous viral genome. Viral components which have been investigated most thoroughly in this regard are nucleic acids, p30 protein, and reverse transcriptase. They are summarized in Table 9. The presence of reverse transcriptase has been used as evidence of viral gene expression in a variety of human malignant tissues due to its relative sensitivity. However, as emphasized above, it is extremely important to examine the specificity of the "reverse transcriptase-like" enzyme detected in these tissues. Reverse transcriptase from human leukemic cells is the only intracellular reverse transcriptase which has been thoroughly studied. Among the criteria of reverse transcriptase described in Table 6, four are most pertinent: (1) high preference of $(dT)_{oligo} \cdot (rA)_n$ over $(dT)_{oligo} \cdot (dA)_n$ as primer-template; (2) ability to efficiently transcribe $(dG)_{oligo} \cdot (rC)_n$ and $(dG)_{oligo} \cdot (rCm)_n$; (3) ability to transcribe heteropolymeric portions of HMW RNA; and (4) antigenic relatedness to reverse transcriptase from known type-C viruses and absence of this relationship to the known cellular enzymes.

(2) Immunological Relationships

The immunological relationships of the intracellular enzyme to known RNA tumor viruses may provide information useful for developing diagnostic or prognostic tests. For example, one might use this property to develop *in situ* immunoprecipitation techniques. This may be especially valuable for human tissues which do not produce detectable extracellular particles. Table 10 shows a summary of the immunological relationships of reverse transcriptases from the primate viruses and from leukemic cells. From these antigenic relationships, the primate viruses can be divided into three distinct groups: the type-C frankly tumorigenic virus group, the type-C endogenous virus group, and the MPMV virus. All three groups are not detectably related to human cellular DNA polymerases α, β, and γ. The reverse transcriptase from some human AML blood cells is closely related to the polymerase from type-C infectious oncogenic viruses. This information may be useful for further exploration and understanding of the viral related information in human cells.

TABLE 9.
RNA Virus Related Information in Human Tumor Cells

RNA tumor virus-like component searched for in human cells	Type-C animal virus probe		
	Primate	Murine	Avian
1. Virus-like DNA in genome	N.D.	+[323]	−[323]
2. Virus-like DNA (product of reverse transcriptase synthesized endogenously "in vitro")	+++[12]	+[12,13]	−[12,13]
3. Viral-like RNA in cytoplasm	+ or +++[12]	+[16]	−[12,16]
4. Viral-like reverse transcriptase biochemical evidence	+++[6,9,108]	+++[6,9,108]	+[6,9,108]
5. Virus-like reverse transcriptase-immunological evidence	+++[8,9]	+[8,9]	−[8]
6. Virus-related antigen (p30)	+++[322]	+[322]	−[322]
7. Virus-like organelle (cytoplasmic particle with density of 1.16 to 1.17, containing reverse transcriptase and virus-like RNA in a functional complex)	+++[12,13]	+[12,13]	−[12]

+++: strong similarity, approaching identity; +: weak, but detectable similarity; −: no detectable similarity; N.D.: not yet detected, still under investigation.

TABLE 10
Immunological Relatedness of DNA Polymerase from the Known Primate Viruses and Cellular DNA Polymerase*

DNA Polymerase from	Antibody to DNA polymerase of			
	SSV-GALV	M-7	MPMV	Human leukocytes
SSV-GALV	+++	0	0	0
Human AML cells, HL23 virus, Hep 12 virus, and C-MuV	+++	0	0	0
M-7 (Baboon endogenous virus)	0	+++	0	0
MPMV	0	0	+++	0
DNA Polymerase α	0	0	0	+++

*+++: More than 90% enzyme activity inhibited by IgG; 0: No inhibition.

(3) Other Related Enzymes

To establish authenticity of intracellular reverse transcriptase, several enzyme activities should be ruled out, for example, cellular DNA polymerase γ and β,[156,158,286] terminal transferase,[291,325,326] and an RNA-dependent but DNA-directed endogenous DNA synthesizing activity.[103,171,172,327] These can be distinguished by a combined use of primer-template specificity, product analysis, and immunological examination. For example, in the presence of Mn^{++}, the purified cellular DNA polymerase β can transcribe $(dT)_{oligo}\cdot(rA)_n$, and $(dT)_{oligo}\cdot(dA)_n$ but not $(dG)_{oligo}\cdot(rC)_n$, while reverse transcriptase can transcribe $(dT)_{oligo}\cdot(rA)_n$ and $(dG)_{oligo}\cdot(rC)_n$, but not $(dT)_{oligo}\cdot(dA)_n$. DNA polymerase β does not transcribe HMW RNA; it is smaller in size than reverse transcriptase, is insensitive to NEM, and is immunologically unrelated to reverse transcriptase. DNA polymerase γ can be distinguished from the viral enzyme by size (it is larger), by its inability to copy viral HMW RNA, and by immunological studies. Terminal transferase simply can be ruled out by observing a reduction in DNA synthesizing

activity with the omission of one or two nucleoside triphosphates and with the lack of DNA synthesizing activity in the presence of primer alone which the transferase utilizes. Also it is much smaller in size than the viral enzyme. The RNA-dependent DNA-directed DNA synthesizing activity is observed in many normal tissues[103,171] and also transformed cells.[327] The distinction of this activity from RNA-directed DNA polymerase depends on careful product analysis by demonstrating the lack of DNA·RNA hybrids in newly prepared DNA products (Table 2), by immunological examination, and by enzyme purification. It is not known which cellular enzyme is responsible for this reaction.

2. Reverse Transcriptase from Cells Producing RNA Viruses

Intracellular reverse transcriptase has been isolated from various cells producing RNA viruses such as Balb/3T3 cells producing (Ki-MuLV)[421] or M-MuSV[287] mouse spleen cells infected by R-MuLV,[100] NC37 cells infected by and producing SiSV-1[99] or GaLV[11], IdU· induced Balb-K/3T3 cells[102] and mammalian cells (human, dog and mink) chronically producing RD114 virus.[435] The enzymes are isolated either from cell homogenates[99,101,287,421] or from the microsomal pellet fraction.[11,100,328] One difficulty in using whole cell or cytoplasmic homogenates is separating cellular DNA polymerases from reverse transcriptase. In most studies with this procedure, the purity of the enzyme is not critical. Recently, Lewis et al.[99] described a method to isolate and separate cellular DNA polymerase α, β, and γ and reverse transcriptase. In this method, cells are homogenized, enzymes are extracted with 1 *M* KCl and nonionic detergent, nucleic acids are removed by fibrous DEAE cellulose, and the enzymes are fractionated on microgranular DEAE cellulose by eluting with 0.05 *M* and 0.3 *M* KCl, respectively. It is interesting to point out that if the buffer contains Triton X-100, almsot all viral enzymes were eluted from the column at 0.05 *M* KCl, but if the buffer does not have Triton X-100, only a portion of the enzyme was eluted from the column at 0.05 *M* KCl. Viral enzyme and DNA polymerase β eluted by 0.05 *M* KCl are fractionated further on a phosphocellulose column with a KCl salt gradient. With this method, the viral enzyme is eluted at 0.2 *M* KCl and is separated well from DNA polymerase β which is eluted at 0.45 *M* KCl. The 0.3 *M* KCl DEAE eluate contains DNA polymerase α and γ. These two enzymes can be fractionated further either by a DNA cellulose column or a hydroxylapatite column. In the DNA cellulose column, DNA polymerase α is eluted at 0.14 *M* KCl, while DNA polymerase γ is eluted at 0.2 *M* KCl. The purity of the reverse transcriptase was not determined. Reverse transcriptase purified by this method has both biochemical and immunological properties closely related (if not identical) to that isolated from extracellular viruses. When a similar scheme was used to fractionate DNA polymerase from PHA stimulated human lymphocytes and from human leukemic cells, all DNA polymerase α, β, and γ were obtained but no reverse transcriptase was detected.[99,329] This finding presents a dilemma regarding the usefulness of this procedure for the isolation of viral enzymes from nonproducing cells. Perhaps, the cells used in the study did not contain reverse transcriptase or contained too little of the enzyme for it to be detected. On the other hand, reverse transcriptase is greatly enriched in the microsomal pellet fraction. In fact, Yang et al.[100] and others[6,318] found that most of the intracellular reverse transcriptase is located in the post-mitochondrial microsomal fraction. The enzymes in the pellet fraction were solubilized in high salt and nonionic detergent and purified by repeated hydroxylapatite chromatography[100] and/or gel filtration.[11,100] The enzyme thus obtained is approximately 800 to 1400-fold enriched and has most of the biochemical and immunological properties of viral reverse transcriptase. This procedure is particularly useful in detecting minute amounts of intracellular reverse transcriptase from cells containing viral information but not producing virus particles (see below) including human leukemic cells. Another method to isolate reverse transcriptase from infected cells is to use affinity chromatographic column as exampled by a recent report by Gerwin et al. By using $(dT)_{oligo}$ cellulose column, they have purified RD114 virus DNA polymerase in one step chromatography and found that the molecular weight of intracellular reverse transcriptase of RD114 virus was 95,000. This is in contrast to reverse transcriptase of purified RD114 virus that has a molecular weight of 70,000. In this study, no aggregate form was observed. This is presumed due to the nature of this particular affinity column or due to the fact that it contained Triton X-100 in their buffer.

3. Is Reverse Transcriptase Present in Normal Tissues?

There are many reports regarding the presence of RNA-dependent DNA polymerase in normal mammalian tissues. However, only one of these studies has definitely demonstrated that the enzyme has the characteristics of viral RNA-directed DNA polymerase. This enzyme was obtained from the placenta-embryo of normal rhesus monkey.[105] It has also been reported earlier that type-C virus can be seen in the placental syncytial trophoblasts of rhesus monkey.[330] The methods used in this study are as follows: The post-mitochondrial microsomal fraction was obtained from a rhesus monkey placenta. The enzyme was solubilized by high salt in the presence of nonionic detergent and processed sequentially through a DEAE cellulose and phosphocellulose column. The enzyme thus obtained had most of the biochemical properties of mammalian type-C virus enzymes, but antigenically it was closely related to reverse transcriptase from M7 virus, an isolate obtained through cocultivation of baboon placental cells with heterologous cells.[62] This virus is now known to be an endogenous virus of baboons. Therefore, reverse transcriptase isolated from placenta is probably a gene product of the endogenous type-C virus of primates and differs from the known oncogenic and infectious primate type-C viruses. It has also been suggested that chicken embryo contains reverse transcriptase activity. This is based on three observations: (1) the endogenous reaction is sensitive to RNase;[104] (2) DNA · RNA hybrids are formed in a short-term endogenous reaction;[331] (3) DNA products are homologous to some RNA isolated from the cytoplasmic pellet fraction.[104] It will be of interest to learn the biochemical and serological properties of the purified enzyme.

In some studies, activity with $(dT)_m \cdot (rA)_n$ was used as evidence for reverse transcriptase in normal cells,[147,148] but as discussed above it is now known that at least two cellular DNA polymerases can also efficiently use $(dT)_m \cdot (A)_n$. These activities were almost certainly due to polymerase γ. A RNase-sensitive endogenous reaction was reported with normal rat tissue culture cells.[103] In this study, the presence of DNA · RNA hybrids was not reported, although small fractions of DNA products were shown to hybridize to RNA isolated from the pellet fraction. However, it is still possible that the RNA used for hybridization is the transcriptional product from DNA existing in the pellet fraction and therefore there is little evidence that the enzymes used RNA as primer-template. A demonstration that the purified enzyme has properties like known viral reverse transcriptase and a demonstration of DNA · RNA hybrids in the endogenous reaction has not been reported. Bobrow et al. observed an RNase-sensitive endogenous DNA synthesizing activity in the microsomal pellet fraction of PHA stimulated lymphocytes.[171] This activity was shown to be an RNA-dependent *DNA-directed* DNA synthesizing activity.[172] The possible presence of a minute amount of RNA-directed DNA polymerase has not been definitely ruled out. The relationship of this activity, if any, to reverse transcriptase activity may be important in determining if reverse transcriptase plays a role in normal cellular proliferation and differentiation.

4. Reverse Transcriptase Activity in Cultured Murine Cells

A new DNA polymerase activity termed Peak A was isolated from the cytoplasmic pellet fraction of two cultured murine cell lines.[102] One is from a clone of BALB/3T3 contact inhibited fibroblasts called A31 and the other is from a subclone of nonproducers obtained by infecting A31 with Ki-MuSV termed KA31. Neither cell line produces virus. Peak A is eluted at approximately 0.22 *M* KCl in a phosphocellulose column with a salt gradient. The amount of Peak A activity from A31 and KA31 was comparable although KA31 cells might have a partial expression of the sarcoma genome[332] and may also contain type-A particles.[102] Peak A activity is similar to viral reverse transcriptase in several ways: (1) the chromatographic pattern is nearly identical. For example, if similar purification procedures were applied to the clone of spontaneously transformed cells continuously producing virus, termed S_2Cl_3, or to extracellular R-MuLV, reverse transcriptase would be eluted at the same KCl molarity; (2) the molecular weight is 70,000 which is the same for reverse transcriptases from mammalian type-C viruses; (3) the enzyme can efficiently use $(dT)_{oligo} \cdot (rA)_n$ as primer-template; and (4) anti-R-MuLV IgG neutralizes 30% of Peak A activity but this is less inhibition than is found with authentic purified R-MuLV reverse transcriptase. It is not known whether this lack of complete neutralization activity is due to a contamination by other cellular enzymes, due to molecular aggregation,[11] or that it is simply a different enzyme. The only distinct difference between Peak A activity and authentic reverse

transcriptase is that Peak A activity is not able to transcribe the rC strand of $(dG)_{oligo} \cdot (rC)_n$ or HMW viral RNA and it is inhibited less by antibody to viral reverse transcriptases. The failure to transcribe 70S RNA and $(C)_n$ does not appear to be due to the presence of an inhibitor. Peak A activity is not DNA polymerase γ as judged by chromatographic behavior, molecular weight, and antigenic relatedness. The biological significance of Peak A activity is not known. It will be of interest to learn if it represents the DNA polymerase associated with intracytoplasmic A particles or whether it is a precursor of reverse transcriptase or even a degraded component of reverse transcriptase.

C. Intracisternal A-particle Associated Reverse Transcriptase

Intracisternal A particles are frequently observed in tumors of many species from birds to man. These particles are observed both in normal and neoplastic tissues[333,334] in mice. Their mode of transmission is not known. It has been speculated that intracisternal A particles represent some sort of viral gene expression and might be an immature type-C virus. However, the latter possibility is unlikely due to the fact that type-A particles contain a group-specific protein which is antigenically unrelated to that of type-C particles. Recently, HMW RNA was found to be associated with intracisternal A particles.[107,218,335] A determination of the relationship between this HMW RNA and that of type-C virus has recently been completed and does not support the idea that they represent immature type-C virus.[107]

The DNA polymerase associated with intracisternal A particles has been initially described by Wilson and Kuff[336] who reported that it can only use $(dT)_{oligo} \cdot (rA)_n$ as primer-template. There was no endogenous activity detectable, and nonionic detergent was not required for the activity. However, if A particles are carefully prepared, an endogenous RNA-dependent DNA polymerase activity, can, in fact, be detected.[106,107] The endogenous reaction is stimulated by nonionic detergent, is sensitive to RNase, and requires all four deoxyribonucleoside triphosphates, divalent cation (Mg^{++} or Mn^{++}), DTT, and monovalent cations for optimum activity. The enzyme has not been purified yet, but it is capable of using $(dT)_{oligo} \cdot (rA)_n$ with high efficiency and $(dG)_{oligo} \cdot (rC)_n$ with less efficiency. $(dT)_{oligo} \cdot (dA)_n$ is not copied. Product analysis of the endogenous reactions from intracisternal type-A particles performed in the presence of actinomycin D shows that the DNA products are initially associated with RNA and 70 to 90% of the purified DNA products can be back hybridized to HMW RNA of the A particles.[107] These findings provide some suggestive evidence that DNA polymerase in type-A particles is very similar to that of authentic reverse transcriptase. It will be of interest to determine if the polymerases of A particles and type-C viruses are immunologically related.

IV. INHIBITORS OF VIRAL REVERSE TRANSCRIPTASE

A. Rationale

A completely specific inhibitor of reverse transcriptase is not yet available. Specificity is relative and often varies with the system measured. For example, a compound can have a Ki value less than 10^{-6} *M* with respect to reverse transcriptase but a Ki of 10^{-3} *M* to another cellular enzyme. The specificity of this compound in a given biological system then depends on the role of the other enzymes in affecting the viability of the cells. If a small depression of the other enzyme's activity lead to a serious cytotoxicity, this compound is naturally not specific in the system under study. Valuable specificity is obtained only when an inhibitor exerts its effect on a particular function in a biological system without inducing cytotoxicity. In searching for specific inhibitors, the enzymes chosen for comparison are important. Many studies have been done with either avian or mammalian viral reverse transcriptase, since these two types of reverse transcriptases have some different characteristics. This should be taken into consideration when comparing results obtained with the two systems. Many studies also claim specificity (or selectivity) of a compound by comparing an inhibitory activity of *E. coli* DNA polymerase to that of reverse transcriptase. This type of study is not relevant to the specificity of the compound in higher organisms. Comparisons are needed between the effect on reverse transcriptase and that on the known DNA polymerases of mammalian cells.

Viral replication and virus-induced transformation can arbitrarily be divided into four steps: formation of the provirus, integration of the provirus, expression of the viral genome, and assembly and release of virus particles. As expected, many studies have shown that reverse transcriptase is required for viral transformation in

tissue culture cells and animals[263,337-341] since it is required for provirus synthesis. After integration of the proviral genome into host DNA, the maintenance and transcription of the proviral DNA does not require reverse transcriptase. Therefore, inhibitors of reverse transcriptase after integration do not have any effect. Nevertheless, prevention of synthesis of proviral DNA theoretically can be useful if additional retransformation events occur.[342] A specific inhibitor of reverse transcriptase might also be a useful tool to determine if reverse transcription is involved in embryogenesis and cytodifferentiation. Finally, a specific reverse transcriptase inhibitor would be helpful in studies directed at reverse transcriptase.

B. Classification

Compounds that inhibit reverse transcriptase can be classified according to their mode of action (see Reference 343). The *compounds* are divided into six groups:

1. Enzyme-binding agents – These include ansamycins (rifamycin SV derivatives[339,344-346] and streptovaricins,[347,348]) calcium elenolate,[349] alkaloid extract of *Narcissus tazetta* L.,[350] Pyran copolymer,[351] and poly(2-O-methyluridylate).[207] None of these compounds is highly specific for reverse transcriptase because they also inhibit other DNA polymerases and RNA polymerase to some degree.

Rifamycin SV derivatives are probably the best studied inhibitors of reverse transcriptase.[343] Following the initial observation of inhibition of reverse transcriptase by some rifamycin SV derivatives by Gurgo et al.,[344] more than 200 derivatives were screened.[345,346] This work was based on an assumption that by modifying some side chains of ansamycin, some compounds specific for reverse transcriptase, (analogous to the specificity of rifamycin to *E. coli* RNA polymerase) would be discovered, but this hypothetical compound was not identified. The rifamycin SV derivatives are grouped into three classes according to potency of inhibition of reverse transcriptase.[14] In a crude virus lysate system, at a concentration of 100 μg/ml, class A compounds are weak inhibitors (inhibit less than 25% of reverse transcriptase activity); class B compounds are moderate inhibitors (inhibit between 25 to 90% of enzyme activity); and class C are relatively potent inhibitors (inhibit more than 90% of the enzyme activity). A similar pattern is obtained when 5 to 10 μg/ml of the compounds are added to the purified enzyme assay system. The Ki value of class C compounds in a purified enzyme system in the absence of nonionic detergent is about 10^{-6} *M*.[218] The class C compounds have been shown to bind to reverse transcriptase both by direct binding studies[218,352] and by kinetic studies.[352] Both tight and loose binding have been described.[218] Two to 14 molecules (with an average of 7 to 8) of inhibitor are bound to 1 molecule of reverse transcriptase.[218,352] The binding is reversible by the addition of nonionic detergent[218] that traps the compounds in micelles.[287] The enzyme activity is completely restored following dissociation from the compound by nonionic detergent. It has been shown that the inhibitor acts at a step before initiation of DNA synthesis. Therefore, conditions which facilitate initiation will abolish the inhibitory activity. Examples of this are the formation of an enzyme template complex, an enzyme-substrate complex or an initiation complex.[218]

Unfortunately, all class C compounds are also potent to moderate inhibitors of cellular DNA polymerases[14,345] and to a lesser degree, RNA polymerase.[218,353] Riva et al. questioned the specificity of class C compounds based on the observation that class C compounds also inhibit some other unrelated enzymes such as glutamate oxaloacetate transaminase, glutamate-pyruvate-transaminase, and alkaline phosphatase.[354] Barlati further argued that the inhibitory activity of class C compounds is due to an exertion of some nonspecific hydrophobic force on the enzymes.[355] However, Gerard et al.[356] reported that the inhibitory activity of class C compounds on reverse transcriptase was specific since they were unable to reproduce the observation made by Riva et al.[354] This discrepancy was probably due to the fact that the concentration of the compounds used by Riva et al.[354] was much higher than that used by Gerard et al.[356]

Although a highly specific inhibitor of rifamycin derivatives has not been found yet, many class C compounds are available.[14,345,346,357] The structure of these compounds and the mechanisms of their action may provide useful information for synthesizing additional derivatives that might be used in developing a specific inhibitor. It appears that both the size of the side chain and the hydrophobicity are important. Thompson et al. recently have synthesized more derivatives.[358] One of them, Rifazone 8_2, was

shown to preferentially inhibit the growth of virus-transformed chick cells in culture.[324] Furthermore, the cytotoxicity is low at the effective concentration.

2. **Substrate analogues** – This includes ara-CTP,[359,360] 2′, 3′-dideoxythymidine triphosphate (ddTTP),[144] and cordycepin triphosphate (Gallo and Wu, unpublished data). All these inhibitors are nonspecific as predicted from their mode of action.

3. **Primer-template analogues** – This group of compounds exerts an inhibitor activity by either tightly binding to reverse transcriptase or competing with primer-template for its binding site. Since the binding of primer-template to nucleic acid polymerase generally is not specific, it is rather difficult to obtain a specific inhibitor of this group. Although some primer-templates such as HMW RNA are specific for viral reverse transcriptase activity, it is not known whether the binding is also specific. Compounds which fall into this group include thymidylate derivatives,[361] polyribonucleotides,[362-365] modified polyribonucleotides such as thiolated polycytidylate and natural RNA,[365,366] and 2′-O-alkylated polyadenylic acids.[370] Among four homopolymers tested, the order of their potency was as follows: $(U)_n >> (G)_n >> (A)_n > (C)_n$.[362] Low concentrations of $(U)_n$ have a higher affinity to viral reverse transcriptase than other cellular DNA polymerases except DNA polymerase γ.[365] However, high concentrations of $(U)_n$ are nonspecific.[363] The size of $(U)_n$ affects its potency. The minimum chain length is 200 nucleotides.[364] Thiolation of $(C)_n$ or some other natural template (even yeast S RNA) improves the potency and the specificity of the polymer as a reverse transcriptase inhibitor.[365,366] Among many partially thiolated polycytidylates, Chandra and Bardos observed that one of them, MPC III (2-6% cytosine bases are thiolated), is relatively a potent inhibitor.[366] The other type of modification obtained by substitution in four and five positions of the base ring and two position on the sugar, such as poly(flurodeoxyuridylic acid), poly(chlorodeoxyuridylic acid), poly(chlorodeoxycytidylic acid), and poly(bromouridylic acid), has been found to significantly alter the inhibitory activity.[367] With increasing information on the nature of the primer in various type-C virus systems, it may be possible to develop analogues which specifically bind to reverse transcriptase.

4. **Template-binding agents** – This group includes actinomycin D,[178,371] chromomycin,[371] pannomycin,[371,372] adriamycin,[371, 373-375] cinerubin,[372] distamycin,[376,377] ethidium bromide,[371,374,378,379] proflavine,[374] tilorone and its cogeners fluroanthene derivatives,[377,380,417] acridine orange, congo red, histone, and protamine.[371] Many of the commonly used antibiotics belong to this group. They inhibit DNA synthesis by binding to some specific bases. For example, actinomycin D binds to guanosine bases while daunomycin, distamycin, and tilorone are adenine-thymidine base-specific.

5. **Divalent cation-binding agents** – By definition, any chelating agent which removes divalent cations required for DNA synthesis could be an inhibitor of DNA polymerase. O-phenanthroline[426] and thiosemicarbazones[160] are examples of these compounds. Apparently, they are not specific inhibitors of any polymerase including reverse transcriptase.

6. **Agents of unknown mode of action** – Streptonigrin is a potent inhibitor of focus formation but a relatively poor inhibitor of reverse transcriptase.[382] Bleomycin inhibits the DNA-dependent DNA synthesis reaction of reverse transcriptase.[383] It inhibits RNA-dependent DNA synthesis only at a high concentration. It is a noncompetitive inhibitor with the DNA primer-template. Other compounds such as 2-oxopropanal,[372] heparin,[371] and silicotungstate[384] are also inhibitors of reverse transcriptase. Heparin seems to be specific for the RNA template since it does not inhibit RNA and DNA polymerases when DNA is used as template.

V. BIOLOGICAL ROLES OF REVERSE TRANSCRIPTASE

A. Reverse Transcriptase and Proviral Synthesis

The only well-defined biological role of viral reverse transcriptase is its requirement for successful viral infection and transformation, i.e., for provirus synthesis. Two types of evidence support this statement. One is the evidence based on studies using reverse transcriptase inhibitors. There is a direct correlation between the inhibition of viral transcriptase activity and viral transformation in culture[339] and of virus induced leukemia in animals[340] by various classes of rifamycin SV

derivatives. The other is evidence based on genetic studies. Hanafusa and Hanafusa isolated a mutant of RSV called RSVα(O) which is defective.[418] This mutant has been shown to lack reverse transcriptase both by biochemical[337] and immunological[338] criteria. Recently, Mason et al.[419] and Verma et al.[263] demonstrated that reverse transcriptase purified from two temperature-sensitive mutants of RSV that are defective in transformation[341] were also temperature-sensitive for DNA synthesis in vitro. Moreover, intracellular viral DNA was not synthesized when duck embryo cells were infected by these two mutants at a nonpermissive temperature (41°C).[422,436] Similarly, Tronick et al. reported that enzyme purified from a temperature-sensitive mutant of R-MuLV (ts29),[271] defective in an early function of virus replication and sarcoma virus helper function, was also temperature-sensitive for DNA synthesis *in vitro*.[434] These findings prove that the reverse transcriptase molecule is required for the synthesis of proviral DNA in vivo and therefore, for transformation and infectivity by type-C virus.

B. Reverse Transcriptase from Normal Tissues

The discovery of transcriptase is basically a fulfillment of the prediction made in Temin's provirus hypothesis.[23] An important by-product of this discovery is the revelation of a new pattern of genetic information transfer, RNA to DNA, which in turn invites new speculations. It is possible that the reverse flow of viral genetic information is only one example of a mechanism more commonly employed. This mechanism could involve repeated cycles of transcription, reverse transcription, and recombination that could lead to somatic mutation and the generation of new information during the course of somatic cell development and differentiation. In fact, these are the basis of Temin's protovirus hypothesis for the generation of viral genes,[385,386] neoplasia[386] and normal development.[386] Similarly, reverse transcriptase has also been proposed to play some role in ribosomal gene amplification[387,388] and in the genesis of various clones of precursors of antibody-producing cells.[389] Experiments to support these proposals, however, are not yet convincing.

These concepts must be supported first by clear evidence that normal cells contain authentic reverse transcriptase. At present, there is no definite evidence that adult tissues of higher organisms (proliferating or nonproliferating) contain authentic reverse transcriptase, although there are some observations which are suggestive.[103,171] There is some evidence indicating that reverse transcriptase is present in embryonic tissues. As described in Section III.B.3, Kang and Temin found RNase-sensitive DNA synthesizing activity in chicken embryonic cells.[104] The endogenous DNA product is found in a hybrid form[331] and the isolated DNA product is able to back hybridize to RNA isolated from the cytoplasmic pellet fraction.[104,331] Mayer et al. isolated a reverse transcriptase activity from rhesus monkey placenta-embryo.[185] This reverse transcriptase activity is immunologically related to viral reverse transcriptase of the M7 virus (isolated from normal baboon placenta and now known to be an endogenous virus), but not to those of known *oncogenic* primate viruses. In addition, type-C-like particles have been visualized in human,[390] chimpanzee,[431] baboon,[391] and rhesus monkey[392] placentas, and virus-like particles have been seen in baboon[393] and mouse[394,395] preimplantation embryos. These findings are in keeping with the idea that the expression of fetal antigens and "virogenes" are related. Furthermore, another viral component, gp70, is found closely linked to lymphoid cell development and differentiation which is mediated through the G_{IX} marker.[432,433]

C. Classification of Reverse Transcriptase

We like to view intracellular reverse transcriptase as two different types, intrinsic and extrinsic. The function of extrinsic reverse transcriptase, as described above, is clear but that of intrinsic reverse transcriptase is not. Concerning reverse transcriptase, the questions that appear to us to be of the greatest interest for the future are related to the intrinsic enzyme. (1) Do all normal cells contain the genome for reverse transcriptase? (2) When is it expressed and what is the function of "intrinsic" reverse transcriptase? (3) Are there functional relationships between intrinsic and extrinsic reverse transcriptase? What the authors present below is hypothetical and is designed to conclude this review by stimulating further studies.

1. Intrinsic Reverse Transcriptase for Development and Differentiation

The meaning of development here is the formation of stem cells for various functions from the

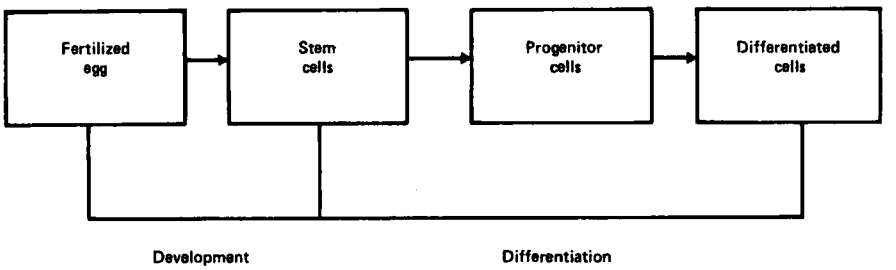

FIGURE 1. Diagrammatic representation of sequence of development and differentiation.

TABLE 11
Interrelationship Between Control of Proliferation and Presence of Reverse Transcriptase, Fetal Antigen, and Viral Antigen

	Control of proliferation	Reverse Transcriptase		Fetal antigen	Viral antigen
		Intrinsic	Extrinsic		
Developing tissues	Relaxed	Present*	N.R.**	Present	Present
Differentiating tissues: early	Stringent	Present	N.R.	N.R.	N.R.
late		?	N.R.	N.R.	N.R.
Malignant tissue	Aberrant, generally relaxed	?	Present	Present	Present

*This does not mean adequate data have been obtained. The terms refer only to what the hypothesis predicts.
**N.R.: Not required.

fertilized egg, and differentiation, as the development of mature functional cells from stem cells (Figure 1). The fertilized egg, by definition, is pluripotent and unable to renew itself. Stem cells are multipotent. They can give rise to more than one kind of differentiated descendant cell. All physiological functions are carried out by the mature cells, such as granulocytes, erythrocytes, sperm, muscle, bone, etc., but they are not able to divide and therefore stem cells must be able to renew themselves to provide a continuous supply of mature cells. The process of differentiation can be further divided into early and late stages. Early differentiation involves the formation of unipotent progenitor cells from stem cells and the late differentiation includes all the processes thereafter (see Figure 1).

During the course of development, the control of proliferation is relatively relaxed (Table 11). Relaxed proliferation is probably required for maximum development. This is the period during which the authors believe intrinsic reverse transcriptase will be used, and in fact, where it has been detected. The exact function, if any, of this reverse transcriptase is, of course, not known. During the development of various types of stem cells, they may require a gene modification and/or amplification. Intrinsic reverse transcriptase might be the catalyst for this purpose. If this is the case, one would predict nonidentity of the genetic information among various types of stem cells. In fact, there is no evidence that all stem cells contain identical genetic information as do germ cells or fertilized eggs. The studies of Gurdon and Woodland[396] showed that at least some must have the complete information contained in the fertilized egg while the studies of Briggs and King showed that nuclear activity changed at or near the onset of gastrulation.[397] Fetal antigens also appear at this state of development. The role of these antigens is not known. They might have some regulatory function or they might be just a by-product of gene expression. In some mouse embryos, type-C particles and viral antigens are frequently detected.[381,395,398] However, these are probably extrinsic reverse transcriptase resulting from congenital infection. It is, of course, not known whether they serve any function in normal development. Upon completion of development of a given system, differentiation of stem cells occurs corresponding to physiological demands, and therefore, more stringent control is required. This type of stringent control is not necessary for the process of development due to the simple environment. In early differentiation when progenitor cells are formed, intrinsic reverse transcriptase might play a role in modifying genetic components for differentiation, such as

generating various clones of progenitors of antibody producing cells. To support this hypothesis, definite proof of the presence of intrinsic reverse transcriptase in normal proliferating cells is required. There is no evidence of fetal antigens at this stage. At the later stages of differentiation, the pattern of cellular proliferation is more confined. This might involve some gene reduction, such as exonucleation by erythrocytes and extrusion of DNA by some lymphocytes, but it would appear unlikely that gene modification occurs at this stage. Intrinsic reverse transcriptase and fetal antigens are, of course, not required at this stage.

2. *Extrinsic Reverse Transcriptase and Carcinogenesis*

Extrinsic reverse transcriptase is introduced into cells upon infection by an oncornavirus. The source of extrinsic reverse transcriptase can be by infection from without or can be produced from within. Its function is to synthesize proviral DNA. Extrinsic reverse transcriptase is normally associated with oncogenic RNA viruses. It may have evolved from intrinsic reverse transcriptase. Extrinsic reverse transcriptase might cause a disturbance in cellular proliferation in two possible ways. First, extrinsic reverse transcriptases might directly cause aberrant gene modification or inappropriate gene amplification. In fact, Loeb and his colleagues have repeatedly observed that viral reverse transcriptase, and probably some human leukemic DNA polymerase as well, have a higher rate of miscopying homopolymers in vitro.[399,400] The rate of infidelity is even higher if heteropolymers or natural primer-templates are used. Second, the provirus might integrate into some specific location such as a "hot spot,"[401] a Tr gene,[402] an oncogene,[381] or some proliferating regulatory genes, resulting in a direct or indirect effect on the stringent control of proliferation through derepression or induction of some relevant genes. In fact, it has been observed that cellular transformation induced by viral infection may occur prior to cell proliferation.[403] It is generally observed that transformed cells have an abnormal mode of cellular proliferation. This abnormality probably is induced by some gene product related to transformation. This is an example of an indirect effect of extrinsic reverse transcriptase.

Once relaxed control of proliferation occurs in adult tissues, it is difficult to convert back to stringent control, since the control element has already been modified during the course of differentiation. However, the reversion of tumor cells to normal cells is not impossible if the genetic lesion is not severe. An example of such conversion is the formation of mature granulocytes from leukemic blast cells in semi-soft agar medium in the presence of a protein factor, termed CFS[404] or in liquid suspension.[405,416] To explain the appearance of fetal antigens in some tumor tissues, one might assume that the gene affecting the regulation of cellular proliferation linked to the virogene is also linked to some fetal antigen genomes (either regulatory or structural). A disturbance of proliferation will then lead to an expression of fetal antigen genes. For example, the S_2 antigen found in some human sarcomas,[406] carcinoembryonic antigen,[407] alpha fetoprotein,[408] and many other placental and fetal proteins.[409,410] are found either in the blood or in the tumor tissues of cancer patients. If these findings are related to a linkage of fetal antigen genes and proliferation genes, it will imply that the expression of fetal antigens in cancer is a by-product rather than a result of the systematic phasing process of embryonic antigens as suggested by Coggin and Anderson.[411] In fact, not all of the fetal antigens are found in all cancers and some are even found in some patients not known to have cancer.[406,412,413]

VI. CONCLUSION

A wealth of information is now available on the biochemical, biophysical, and immunological properties of various reverse transcriptases. The enzyme has been of great help for the identification and quantitation of virus, for preparing molecular probes, as an indication of virus in tissues not known to be producing virus; and, of course, for providing major information for our understanding of the replication of these viruses. The origin and the role of the enzyme is clear in virus-infected cells. What is unclear and potentially more important relates to the possibility that reverse transcriptase, distinct from that of infecting viruses, may have a role in development and differentiation. The authors of this review call this "intrinsic" reverse transcriptase, and they think that it is involved in gene modification and/or gene amplification during development. The authors propose that extrinsic reverse transcriptase, besides being able to synthesize provirus, may cause abnormal cell proliferation either by affecting gene modification or gene amplification, or by integrating the provirus into the host

chromosomes at some specific location. Support for some of these concepts will require, among many other studies, definite proof of the existence of intrinsic reverse transcriptase in a variety of cells and especially its identification in normal adult cells.

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GLOSSARY

AMV	– Avian myeloblastosis virus.
CCC	– An isolate of feline endogenous virus.
CSV	– Chicken syncytial virus.
FeLV	– Feline leukemia virus.
FeSV	– Feline sarcoma virus.
G-FeLV	– Gardner strain of FeLV.
G-FeSV	– Gardner strain of FeSV.
R-FeLV	– Richard strain of FeLV.
R-FeSV	– Richard strain of FeSV.
T-FeLV	– Thelian FeLV.
GaLV (SEATO)	– Gibbon ape lymphosarcoma virus isolated from SEATO (Thailand) gibbon ape colony.
GBr-1, GBr-2, and GBr-3	– GaLV-like virus isolated from brain tissues of three gibbon apes obtained from Southeast Asia.
HaLV	– Hamster leukemia virus.
HaSV	– Hamster sarcoma virus.
M – 7	– Baboon endogenous virus isolated from placenta.
MMTV	– Mouse mammary tumor virus.
MPMV	– Mason-Pfizer monkey virus.
H-MuSV	– Harvey strain of murine sarcoma virus.
F-MuLV	– Friend strain of murine leukemia virus.
K-MuLV	– Kirsten strain of MuLV.
K-MuSV	– Kirsten strain of MuSV.
M-MuLV	– Moloney strain of murine leukemia virus.
M-MuSV	– Moloney strain of murine sarcoma virus.
R-MuLV	– Rauscher strain of MuLV.
C-MuV	– Caroli murine virus.
PK (15) virus	– Endogenous type-C virus isolated from porcine cell line PK.[15]
RaLV	– Rat leukemia virus.
RAV	– Rous associated virus.
RAV-1	– Strain no. 1 of Rous associated virus.
RAV-2	– Strain no. 2 of Rous associated virus.
RD-114	– Feline endogenous virus induced by and grown in human rhabdomyosarcoma (RD) cells.
REV	– Reticuloendotheliosis virus.
REV-T	– Reticuloendotheliosis (strain T).
RSV	– Rous sarcoma virus.
B77	– B77 strain of RSV.
B-RSV	– Bryan high titer strain of RSV.
RSV (RAV – 0)	– RSV with pseudotype Rous associated virus-0 (RAV-O).
PR-RSV	– Prague strain of RSV.
Pr-RSV/A	– PR-RSV subgroup A.
PR-RSV/B	– PR-RSV subgroup B.
PR-RSV/C	– PR-RSV with subgroup C.
SR-RSV	– Schmidt Ruppin strain of RSV.

SR-RSV/A	– SR-RSV subgroup A.	**LMW**	– Low molecular weight.
SiSV SiLV	– Simian sarcoma leukemia virus.	**NEM**	– N-ethylmaleimide.
		DMSO	– Dimethylsulfoxide.
TD SNV	– Trager duck spleen neurosis virus.	**DTT**	– Dithiothreitol.
		IgG	– Immunoglobulin G.
SV40	– Simian virus 40.	**NC-37 cells**	– A normal human lymphoblastoid culture cell.
HMW	– High molecular weight.		

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